Supramolecular Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713649759

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To cite this Article Sugimoto, Naoki , Sasaki, Muneo and Tanaka, Atsushi(1993) 'Relationship between catalytic activity and secondary structure of a hammerhead ribozyme: A study using thermodynamic parameters for RNA structure prediction', Supramolecular Chemistry, 2: 2, 99 — 102
To link to this Article: DOI: 10.1080/10610279308038303
URL: http://dx.doi.org/10.1080/10610279308038303

SUPRAMOLECULAR CHEMISTRY, Vol. 2, pp. 99–102
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Relationship between catalytic activity and secondary structure of a hammerhead ribozyme: a study using thermodynamic parameters for RNA structure prediction

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(Received July 26, 1992)

The stabilization energy for the secondary structures of wild-type hammerhead and mutant ribozymes has been calculated at different salt conditions and temperatures by using the thermodynamic parameters for RNA structure prediction. The most stable structure at each condition has been searched and the obtained secondary structure is compared with the structure suggested phylogenetically or experimentally. The results indicate that the hammerhead-type secondary structure of the ribozyme and its reactivity correlate with each other. The multibranched loop containing the self-cleavage site of the ribozyme particularly should be a key structure in the hammerhead ribozyme reaction. The predicted secondary structures also suggest that the reactivity of the hammerhead ribozyme should be very much lower at 10°C than that at 37°C.

INTRODUCTION

There has been great interest in ribozymes, that are RNA catalysts, reaction that cleave both RNA and DNA without any protein enzyme.[1–6] A small ribozyme having a consensus structure termed 'hammerhead' is especially interesting.[7,8] A design of small ribozymes which were able to cleave RNA substrates by recognizing sequences of only 9–15 nucleotides was previously reported.[9–11] It was suggested that the stability of secondary structures in the ribozymes was important for the cleavage of target RNAs.[11,12] However, despite the importance, little is known about the relationship between a ribozyme structure and its function.

As a first step in the investigation of the relationship, it should be useful to predict the secondary structure of a ribozyme from its primary sequence. Several methods have been reported for the prediction of RNA secondary structures: a phylogenetic comparison,[13] an experimental prediction,[14] and computer predictions.[15–17] The computer predictions are based on searching the secondary structure of RNA with a minimum free energy. One of the main problems with the computer prediction is the use of ambiguous free-energy parameters for the formation of regions with unpaired nucleotides such as an internal loop, a bulge, and a hairpin loop.[17] Therefore, the computer prediction is expected to be successful if the parameters are improved at different salt and temperature conditions. Recently, we developed a calculation method based on new nearest neighbour parameters of RNAs with unpaired nucleotides, and reported the stabilization energy of complexes between some ribozymes and RNA substrates in 1 mol dm^{-3} NaCl buffer.[18]

In this work, the stabilization energy for the secondary structures of a hammerhead ribozyme was calculated at different salt conditions and temperatures by using the thermodynamic parameters for RNA structure prediction, and searched for the most stable structure at each condition. The secondary structure obtained has been compared with the structure suggested phylogenetically and experimentally. The comparison has provided useful information about similarities and differences in the structures and the relationship between the secondary structure and the reactivity of the ribozyme.

METHODS

The enthalpy, entropy, and free-energy changes for the formation of the secondary structure of RNA were

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calculated by using nearest neighbour parameters for RNA sequences as described previously.[18] Our calculation method is based on the method of Zucker and Stiegler[15] which was used to predict secondary structures of RNA. Energetically stable secondary structures of RNA were searched from the matrix of the energy values obtained by the thermodynamic calculation for the possible base pairings. Our new parameters obtained by UV melting curves of oligonucleotides[19] were used especially for internal loop, bulge, and hairpin structures at different salt conditions and temperatures. The free energy of the loops with more than 30 nucleotides was calculated using the temperature-dependent equation of Jacobson and Stockmayer.[20] Multibranched loop parameters were calculated by using internal loop parameters instead of using the previous calcultion.[21]

The program for calculating energeties of RNA runs in MS-DOS environment with NEC PC-9801 computer series. The maximum number of nucleotide sequences to be treated in the program is about 400. Pseudoknots were not included in the program. The secondary structures obtained and their energy values were compared with those suggested experimentally and phylogenetically.

RESULTS AND DISCUSSION

Our new method for predicting the secondary structures of RNA has three improved features. First, our program can predict RNA secondary structure at arbitrary temperatures. Second, the program can predict not only the most stable structure but also the metastable structures of RNA. The possibility of getting the chemical equilibrium between the most stable and other stable structures is very important because not only the most stable but also the metastable structures may be biologically important. Finally, the program can calculate the free energy for the folding structure not only in Na^+ aqueous solutions but also in the presence of Mg^{2+}. A divalent cation like Mg^{2+} is necessary for the self-splicing and ribozyme reactions of the group I intron from *Tetrahymena thermophila* and the RNA ribozymes.[1-11] The comparison of the predicted secondary structures and their thermodynamic values in the presence of Mg^{2+} with those in the presence of Na^+ is very useful for investigating the reactivity of the ribozymes.

A hammerhead ribozyme is known to be a catalytic RNA and has a self-cleaving domain to generate 5′-hydroxy and 2′,3′-cyclic phosphate termini.[8] This type of ribozyme exists in a variety of plant viroid and virusoid RNAs.[22-24] A secondary structure model of the domain of the hammerhead ribozyme suggested experimentally[25] is shown in Figure 1. The ribozyme cleaves itself in the presence of Mg^{2+} without any protein enzyme.[8-11] The structure in Figure 1 has been considered to be a favourable structure because of its self-cleavage reaction.[8-11] The secondary structure termed 'hammerhead' consists of three helices and two hairpin loops. The self-cleavage site is between C6 and A7 in the multibranched loop. Thirteen boxed nucleotides are considered to be a catalytic domain termed the 'conserved sequences.[8-11]

Zucker *et al.*[15,21,26] developed the MulFold program for obtaining the most stable secondary structure of RNA, as described earlier. The program gives the same secondary structure in any conditions such as in the presence of Mg^{2+} or Na^+. Figure 2 shows the most

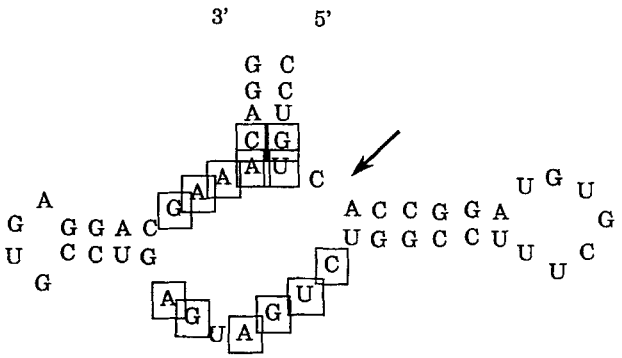

Figure 1 Secondary structure of the hammerhead ribozyme suggested experimentally. The arrow shows the self-cleavage site, between C6 and A7, in the multibranched loop. Boxed nucleotides are considered to be a catalytic domain termed the 'conserved sequence.'

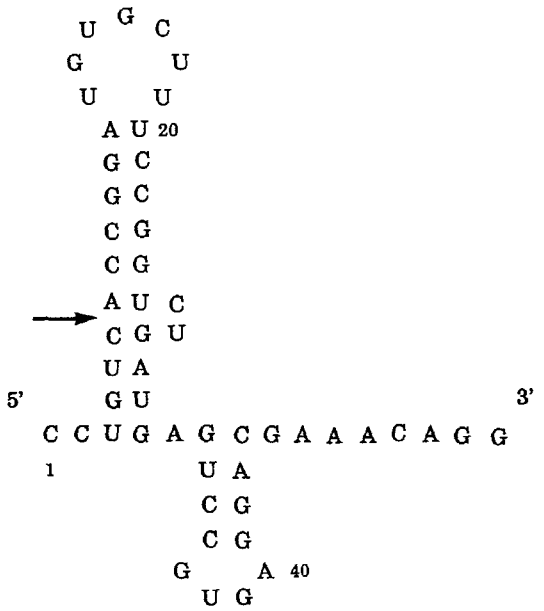

Figure 2 Secondary structure of the hammerhead ribozyme obtained by the MulFold program. The arrow shows the self-cleavage site, between C6 and A7, in the multibranched loop.

stable secondary structure of the ribozyme calculated using the program. The obtained structure is very different from the hammerhead-type structure and consists of two hairpin loops, two stems, and long, unpaired terminal nucleotides. In the structure, there is no multibranched loop which was suggested experimentally. The self-cleavage site between C6 and A7 is in the stem region, that is, the double-helix region, which is also very different from the case shown in Figure 1. The reason for the difference between the structures may be due to using ambiguous thermodynamic parameters in the program for the formation of regions with unpaired nucleotides such as an internal loop, a bulge, or a hairpin loop.

In this work, an energetically minimized secondary structure of the hammerhead ribozyme was searched at 6 mmol dm^{-3} Mg^{2+} and 37°C at which the ribozyme reaction was usually performed. The structure obtained is shown in Figure 3 with the stabilization energy ΔG^0. The structure is very similar to the secondary structure suggested experimentally in Figure 1. The exception is only a base-pair formation of U30 and A47 in Figure 3. The base-pair formation suggests that in Mg^{2+} aqueous conditions the symmetrical loop as shown in Figure 3, is more stable than the asymmetrical loop shown in Figure 1, and the suggestion supports the result obtained in the case of RNA oligonucleotides.[27] The self-cleavage site is between C6 and A7 in the multibranched loop, which is consistent with the case in Figure 1. The similarity between the structures suggested experimentally and calculated by our method indicates our parameters and program are very useful for the secondary structure prediction of RNA.

Ruffer *et al.*[28] reported that the reactivity of the hammerhead ribozyme decreased with the mutation of the conserved sequence which was considered to play an important role in the catalytic ribozyme reaction. We have searched energetically minimized

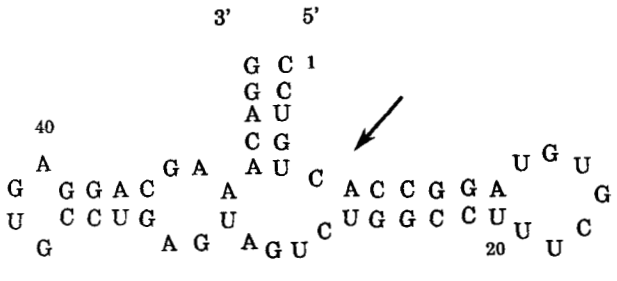

Figure 3 Energetically minimized secondary structure of the hammerhead ribozyme at 6 mmol dm^{-3} Mg^{2+} and 37°C. ΔG° is the stabilization energy of the secondary structure. The arrow indicates the self-cleavage site, between C6 and A7, in the multibranched loop.

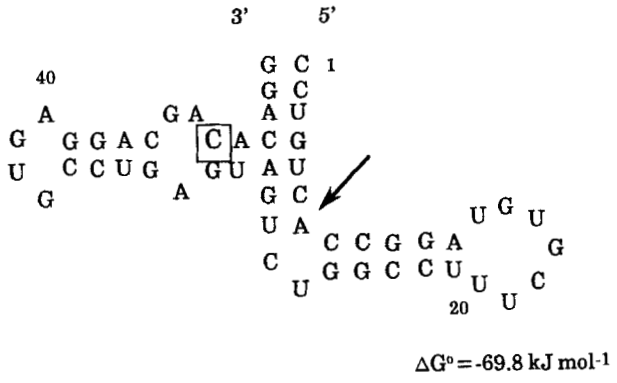

Figure 4 Energetically minimized secondary structure of the mutant hammerhead ribozyme (A47–C) at 6 mmol dm^{-3} Mg^{2+} and 37°C. The arrow indicates the self-cleavage site, between C6 and A7, in the multibranched loop.

secondary structures of the A47–C mutated hammerhead ribozyme at 6 mmol dm^{-3} Mg^{2+} and 37°C. The structure obtained is shown in Figure 4 with the stabilization energy ΔG^0. This structure is very different from the hammerhead type shown in Figures 1 and 3. In Figure 4, there is no multibranched loop, which is the greatest difference when comparing Figures 1 and 3. The self-cleavage site between C6 and A7 is in the stem region, which was observed in Figure 2. The stabilization energy of the mutant ribozyme in Figure 4 is -69.8 kJ mol^{-1}, and the structure is more stable than that in Figure 3 by about 3 kJ mol^{-1}. This suggests that this secondary structure of the mutant ribozyme hardly changes the active hammerhead-type structure, and it leads to the decrease of the ribozyme reactivity. It is very interesting that only a one-point mutation in the conserved sequence leads to the dramatic change of the secondary structure of the ribozyme and then the lower reactivity.

Finally, energetically minimized secondary structures of the hammerhead ribozyme at 6 mmol dm^{-3} Mg^{2+} has been searched at 10°C. The structure obtained is shown in Figure 5 with the ΔG^0 value. The obtained structure is very different from the hammerhead-type structure in Figures 1 and 3. In Figure 4, there is not the multibranched loop. The self-cleavage site between C6 and A7 is in the stem region, which was observed in Figure 4. The stabilization energy of the secondary structure in Figure 5 is -97.8 kJ mol^{-1}. The results indicate that the structure of the ribozyme at 10°C is more stable than that at 37°C (Fig 3). This suggests that the ribozyme forms the very stable secondary structure with very low reactivity which hardly changes the active hammerhead-type structure.

In conclusion, this work indicates that the hammerhead-type secondary structure of the ribozyme and its reactivity correlate with each other, and the multibranched loop containing the self-cleavage site should

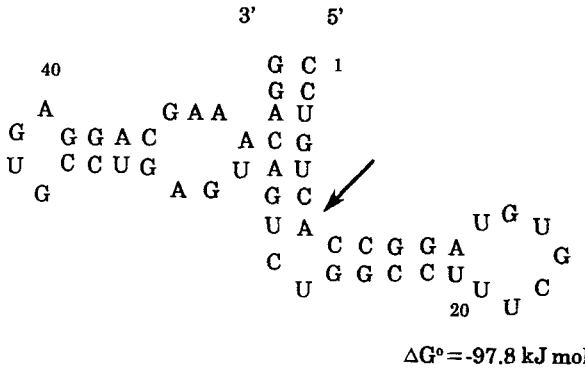

Figure 5 Energetically minimized secondary structure of the hammerhead ribozyme at 6 mmol dm^{-3} Mg^{2+} and 10°C. The arrow indicates the self-cleavage site, between C6 and A7, in the multibranched loop.

be a particularly key structure in the hammerhead ribozyme reaction. This work predicts from the obtained secondary structures that the reactivity of the hammerhead ribozyme should be very much lower at 10°C than that at 37°C.

ACKNOWLEDGMENT

This work was supported in part by the Grant-in-Aid for Science Research on Priority Areas from the Ministry of Education, Science and Culture, Japan.

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