Hammerhead Ribozyme Mechanism: A Ribonucleotide 5′ to the Substrate Cleavage Site Is Not Essential†

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Received June 10, 1999

ABSTRACT: Three hammerhead ribozymes with triplet specificities for cleavage 3′ of CUC, GUC, and GUA have been evaluated for their sensitivity to the substitution of thymidine or 2′-deoxyuridine at central nucleotide position 16.1 in the substrate triplet. All three ribozymes cleaved their respective substrates, containing uridine or the modifications, with comparable rates. This indicates that the 2′-hydroxy group at position 16.1 is not essential for activity even though X-ray structure analysis shows it participates in H-bonding interactions. These H-bonds were considered to be of functional significance because an earlier report had provided data that thymidine at position 16.1 is deleterious for catalytic activity [Yang, J.-H., Perreault, J.-P., Labuda, D., Usman, N., and Cedergren, R. (1990) *Biochemistry 29*, 11156−11160].

Several X-ray structural analyses of the hammerhead ribozyme describe not only the ground state of the ribozyme−substrate complex but also configurations approaching the transition state (*1*−*4*). These structural data indicate a local conformational change in the transition state around the cleavage site which is consistent with the stereochemical outcome of the reaction, observed in solution. The structures determined by Scott et al. (*2, 3*) ascribe a particular role to the 2′-hydroxy group of uridine 16.1 in the cleavable triplet GUC, by forming a hydrogen bond to N-3 of adenosine in position 6, and, in the transition state, an additional hydrogen bond to O-4′ of cytidine 17.1. These interactions have been considered important because a previous study had reported that thymidine at position 16.1 reduces the catalytic efficiency of a CUC cleaving ribozyme by a factor of 180 (*5*). We became interested in the inhibitory effect of thymidine while performing in vitro selection experiments with hammerhead ribozymes and therefore repeated the kinetic characterization with the reported ribozyme and substrate. To our surprise, cleavage of the thymidine-containing substrate was as efficient as the uridine-containing substrate. The study was then extended to two other ribozyme constructs and substrates of different sequences. In all the cases, it was observed that ribozymes efficiently cleaved substrates containing thymidine or 2′-deoxyuridine at position 16.1 when compared to uridine at the same position. These observations indicate that contrary to the interpretation of the X-ray structure analyses, the 2′-hydroxy group of U16.1 in the hammerhead ribozyme substrate is of no functional importance.

MATERIALS AND METHODS

Materials. Substrates and ribozymes were synthesized on an Applied Biosystems 394A DNA synthesizer. Phosphoramidites were obtained from Proligo Biochemie (Hamburg, Germany), except for those of 2′-fluorouridine and 2′-aminouridine which were supplied by NeXstar Inc. (Boulder). Purification of oligonucleotides was done as reported previously (*6, 7*). [γ-^{32}P]ATP (specific activity approximately 5000 Ci/mmol) was from Amersham Buchler GmbH. T4 polynucleotide kinase and 10× reaction buffer (700 mM Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT) were purchased from Biolabs. Siliconized Eppendorf tubes were obtained from Biozym Diagnostik (Germany). Cellstar Micro-Plate TC sterile was from Greiner Labortechnik. X-ray films (X-OMAT XAR-5) were purchased from Kodak. Radioanalytical scanning was performed on a Fuji BAS 2000 Bio-Imaging analyzer.

Cleavage Kinetics. Single-turnover (STO)[1] cleavage rates $k_{cat'}$ and $K_{m'}$ were determined with 5′-^{32}P-labeled substrate. Rates were determined from the initial phase by increasing the ribozyme concentration until a plateau was reached. Stock solutions of 5′-^{32}P-labeled substrate and nonradiolabeled ribozyme in 50 mM Tris-HCl, pH 7.5, were heated separately at 90 °C for 1 min and cooled to 25 °C for 15 min, followed by addition of $MgCl_2$ to a final concentration of 10 mM. Reactions were initiated by the addition of ribozyme solution to the solution of substrate, and were carried out in a final volume of 50 μL at 25 °C. Final concentrations of ribozymes ranged from 50 to 500 nM, and substrate concentration was 25 nM. MTO reactions to determine k_{cat} and K_m were conducted at pH 7.5 and 25 °C with the ribozyme concentration ranging from 2.5 to 25 nM and that of substrate from 50 to 500 nM. For some ribozymes, pH and temperature deviated from these standard settings. These are indicated in the tables. Complete progress curves of cleavage were determined with 25 nM substrate and 500 nM ribozyme. Aliquots were removed for analysis at different time points, and the reaction was quenched by addition of an equal volume of stop-mix (7 M urea, 50 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue) in Micro-Plates with

† This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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[1] Abbreviations: STO, single turnover; MTO, multiple turnover; fU, 2′-deoxy-2′-fluorouridine; aU, 2′-deoxy-2′-aminouridine.

10.1021/bi991332n CCC: $18.00
Published on Web 08/05/1999

Table 1: Michaelis−Menten Parameters for Ribozyme I[a]

nucleotide at position 16.1	K_{cat} (min^{-1}) [reported in ref *5*]	K_m (μM) [reported in ref *5*]
U[b]	1.20 [1.2]	0.372 [0.7]
T[c]	1.56 [0.12]	0.388 [7.4]

[a] MTO kinetics with 10 mM $MgCl_2$, Tris-HCl, pH 7.5. [b] At 30 °C. [c] At 37 °C.

Table 2: Rate Constants for Ribozymes[a]

	$k_{cat'}$ (min^{-1}) for ribozymes		
nucleotide at position 16.1	I	II	HH16
U	3.87[b] 2.01	0.82	0.74
dU	3.32[b] 2.18	0.99	ND
T	2.72[c] 2.36	ND	0.49
fU	ND[e]	0.91[d]	0.66
aU	ND	0.88	ND

[a] STO kinetics with 10 mM $MgCl_2$, Tris-HCl, pH 7.5, at 25 °C unless stated otherwise. [b] Determined at 30 °C. [c] Determined at 37 °C. [d] Determined at pH 7.0. [e] ND, not determined.

subsequent snap-cooling on ice. Substrate and products were separated on a 20% polyacrylamide/7 M urea denaturing gel and analyzed by the Bio-Image Analyzer. The extent of cleavage was determined from measurements of radioactivity in the substrate and the 5′ product bands. Data were fitted to the Michaelis−Menten and Eadie−Hofstee equations by KaleidaGraph (Synergy Software, Reading, PA). Values given in Tables 1 and 2 are the average of at least 2 determinations with a deviation of ≤10% for $k_{cat'}$ and ≤45% for $K_{m'}$ values.

RESULTS

The ribozyme and substrate sequences for the first experiments were identical to those described by Yang et al. (*5*) (Scheme 1, ribozyme I). MTO kinetics were conducted under the conditions reported there; i.e., cleavage 3′ of $CU^{16.1}C^{17}$ was carried out at 30 °C and that 3′ of $CT^{16.1}C^{17}$ at 37 °C. (Table 1). The value obtained for k_{cat} for cleavage of CUC was 1.20 min^{-1}, and the K_m value was 0.372 μM. These values compare well with those published as 1.2 min^{-1} and 0.7 μM. However, values for the cleavage of CTC differed markedly. Whereas we find k_{cat} to be 1.56 min^{-1}, the reported value is 0.12 min^{-1}, and our K_m value of 0.388 μM is much smaller than the reported value of 7.4 μM. To determine the rate of the chemical step, STO kinetics were performed also at 30 and 37 °C. Values for $k_{cat'}$ of 3.87 min^{-1} for CUC at 30 °C and of 2.72 min^{-1} for CTC cleavage at 37 °C were obtained (Table 2). As these rates were quite high, the kinetic experiments were repeated at 25 °C to determine them more accurately. Under these conditions, the rates were 2.01 min^{-1} for cleavage 3′ of CUC, and 2.36 min^{-1} for 3′ of CTC. To eliminate an influence of the methyl group of thymidine, the cleavage rate 3′ of $CdU^{16.1}C^{17}$ was also determined and was found to be 3.32 and 2.18 min^{-1} at 30 and 25 °C, respectively.

Ribozyme HH16 (*8*) and ribozyme II (Scheme 1) were also tested to probe the effect of sequence on the catalytic activity toward substrates containing thymidine or 2′-deoxyuridine at position 16.1. HH16 cleaves 3′ of $G^{16.2}U^{16.1}C^{17}$, which is very similar to the substrate triplet of ribozyme I. Thymidine had a minor effect on $k_{cat'}$ with 0.49 min^{-1} as compared to 0.74 min^{-1} for the cleavage of GUC (Table 2). To test other 2′-modified nucleotides, we also evaluated another substrate containing the triplet $G^{16.2}fU^{16.1}C^{17}$ as the cleavable triplet. A cleavage rate of 0.66 min^{-1} was found for this triplet. Ribozyme II which cleaves 3′ of $G^{16.2}U^{16.1}A^{17}$ was investigated to see whether there was an effect of dU on the cleavage 3′ of A, as compared to the cleavage 3′ of C (Table 2). The cleavage rate for the $G^{16.2}dU^{16.1}A^{17}$ triplet was 0.99 min^{-1} as compared to 0.82 min^{-1} for the cleavage of GUA. Interestingly, cleavage of the substrate containing a $G^{16.2}fU^{16.1}A^{17}$ triplet was too fast to measure reliably at pH 7.5 and was therefore reexamined at pH 7.0. The value of 0.91 min^{-1} extrapolates to approximately 2.5 min^{-1} at pH 7.5 (*9*, *10*). Ribozyme II was also tested with a substrate containing aU at position 16.1 which was cleaved at 0.88 min^{-1} .

MTO kinetics were also performed for ribozyme II with the $G^{16.2}fU^{16.1}A^{17}$ and $G^{16.2}aU^{16.1}A^{17}$ substrates (Table 3). The data indicate only minor differences in the Michaelis−Menten parameters. The extent of cleavage was also determined for the $G^{16.2}fU^{16.1}A^{17}$ substrate, at pH 7.0 (Figure 1). Approximately 75% of substrate was cleaved within 2 min with a single rate constant which is comparable to the cleavage of the $G^{16.2}U^{16.1}A^{17}$ substrate (data not shown).

Products of all cleavage reactions of substrates with modified nucleotides at position 16.1 had the same gel

Scheme 1

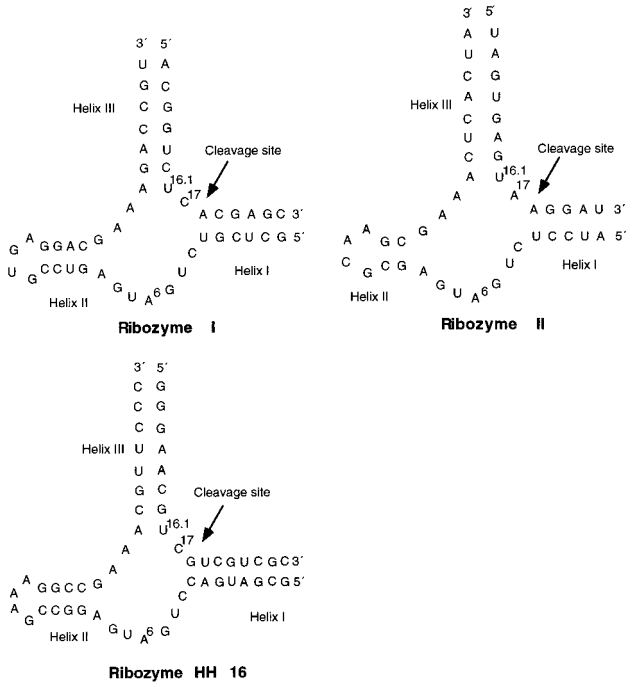

Table 3: Michaelis−Menten Parameters for Ribozyme II[a]

nucleotide at position 16.1	$k_{cat'}$ (min^{-1})	K_m (nM)
U	0.84	37.9
fU	1.00	37.1
aU	0.81	47.1

[a] MTO kinetics with 10 mM $MgCl_2$, Tris-HCl, pH 7.5, at 25 °C.

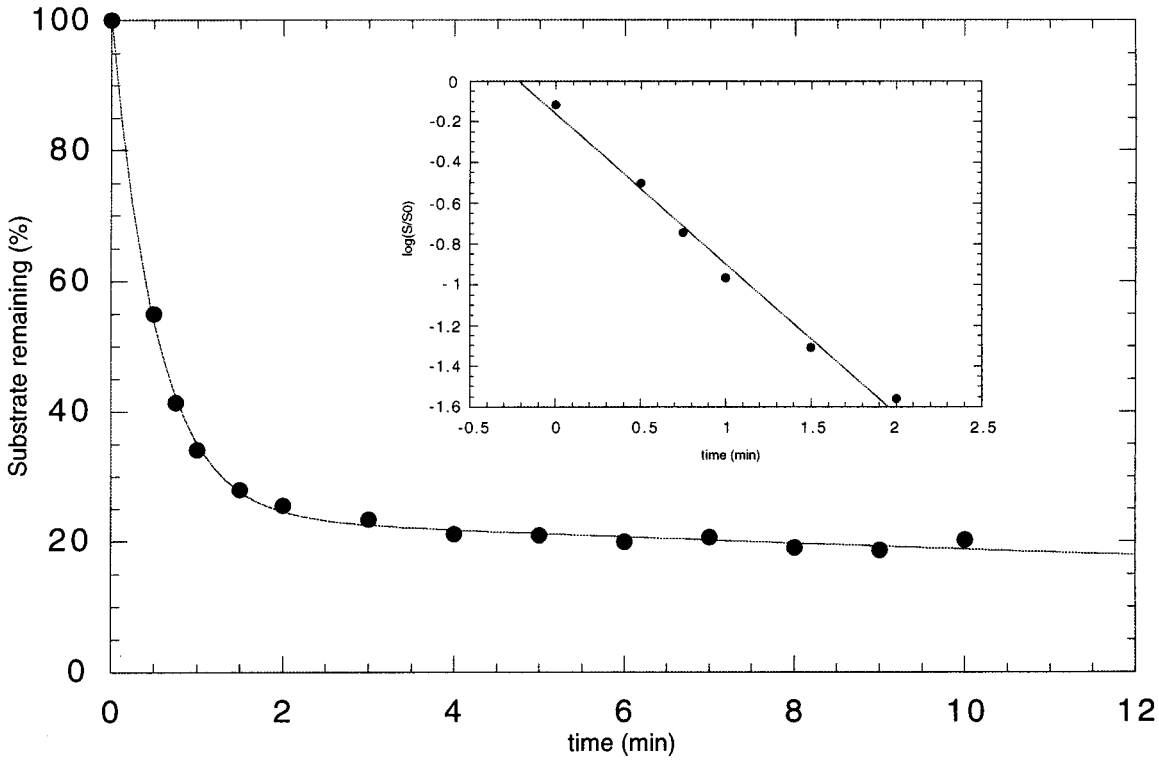

FIGURE 1: Time course of STO cleavage of substrate $GfU^{16.1}A^{17}$ with ribozyme II. Conditions were 10 mM $MgCl_2$, Tris-HCl, pH 7.0, at 25 °C with 25 nM substrate and 500 nM ribozyme. Insert: first phase of the reaction in a semilogarithmic plot.

mobility as cleavage of the uridine-containing substrate, indicating that the reaction resulted in a terminal 2′,3′-cyclic phosphate.

DISCUSSION

The structure−function relationship of the hammerhead ribozyme has been discussed in several recent reviews (*11*−*15*). X-ray structural data have provided detailed atomic resolution to gain insight into intramolecular interactions (*1*−*4*). The structures of the ribozyme−substrate complex approaching the transition state are of particular interest, as it is generally accepted that a conformational change has to occur for the reaction to proceed. In the ground-state structure, uridine 16.1 has been shown to form a H-bond through its 2′-hydroxy group and N-3 nitrogen of A6 (*2*). In a structure approaching the transition state, the distance of this H-bond is reduced and an additional H-bond is formed to the O-4′ furanose oxygen of C17 (Figure 2) (*3*). These H-bonds are considered important, particularly as Yang et al. (*5*) have demonstrated that the replacement of U16.1 in a hammerhead ribozyme substrate by thymidine results in a decrease of k_{cat} by a factor of 22 and an 8-fold increase of K_m, thereby reducing the catalytic efficiency by a factor of 180. Thus, the 2′-hydroxy group at position 16.1 seems to be important for the organization of the ground-state structure, and possibly, even more importantly, also in the stabilization of the transition state.

We repeated the MTO kinetic characterization of the ribozyme construct of Yang et al. (*5*) for cleavage 3′ of CUC and CTC under the reported conditions (Table 1). These authors had found considerable differences in k_{cat} and K_m for these two substrates. Although we could essentially confirm the data for CUC cleavage, the values we obtained for CTC cleavage differed considerably from those published,

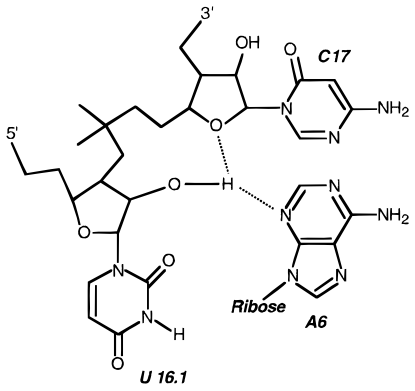

FIGURE 2: Schematic representation of hydrogen bonds formed by the 2′-OH group of uridine 16.1 according to Scott et al. (*3*).

in terms of both k_{cat} and K_m. These data indicated only a minor effect by the presence of T at the central position of the substrate triplet. As the rate of cleavage determined under MTO does not necessarily represent the chemical step which would be important for any mechanistic interpretation of the cleavage reaction, we measured the cleavage rate under STO (Table 1). Again, the cleavage rates were determined at 30 and 37 °C, for the two substrates. As the rates were unexpectedly fast, these determinations were repeated at the more conventional temperature of 25 °C to obtain more precise values and to make them comparable to other ribozyme data. The results confirmed that the presence of thymidine in the substrate did not affect the cleavage rate appreciably. We also evaluated cleavage by ribozyme I of substrates containing 2′-deoxyuridine at position 16.1. Under STO conditions, the $k_{cat'}$ values at 30 and at 25 °C were very close to the observed cleavage rate 3′ of the CUC triplet, thus eliminating any steric effect by the 5-methyl group. Thus, our results are not in agreement with the published

data on the inhibitory effect of T for MTO, and the STO data confirm this conclusion. We cannot explain the reason for this discrepancy at present.

The original data by Yang et al. (*5*) have been used for the analysis of the X-ray data to establish the importance of the 2′-hydroxy group at position 16.1 for the hammerhead ribozyme with a substrate containing a GUC triplet, even though the original kinetic data were obtained for a CUC triplet. To confirm the generality of our observations with ribozyme I, we also evaluated the requirement of the 2′-OH group using the well-characterized hammerhead ribozyme HH16, which efficiently cleaves 3′ of GUC (*8, 9*). It was found that cleavage activity was somewhat reduced when thymidine was introduced. However, with fU the rate was almost identical to that with uridine. Therefore, no functional role can be assigned to ribose at this position.

There was still the possibility that there was a sequence specificity of this effect for the cleavage 3′ of $U^{16.1}C^{17}$, two nucleotides which are common to the substrates of ribozymes I and HH16. The triplet $G^{16.2}U^{16.1}A^{17}$ was therefore chosen as an alternate substrate for ribozyme II. Cleavage of $G^{16.2}dU^{16.1}A^{17}$ was slightly faster than that of $G^{16.2}U^{16.1}A^{17}$ with $k_{cat'}$ 0.99 min^{-1} vs 0.82 min^{-1}, clearly indicating a lack of inhibitory effect due to the presence of 2′-deoxyuridine. This ribozyme presented an opportunity to test other 2′-modified uridines as part of the cleavable triplet to identify conformational contributions to cleavage susceptibility. Thus, fU and aU were incorporated into the GUA triplet. The cleavage rate for $G^{16.2}fU^{16.1}A^{17}$ was too fast to accurately determine at pH 7.5 and was therefore measured at 7.0. A $k_{cat'}$ value of 0.91 min^{-1} was found which extrapolates to 2.5 min^{-1} at pH 7.5 (9). Cleavage of $G^{16.2}aU^{16.1}A^{17}$ was comparable to that of GUA. Thus, the fU-containing triplet is cleaved most efficiently.

Ribozyme II was also tested for cleavage of $G^{16.2}U^{16.1}A^{17}$, $G^{16.2}fU^{16.1}A^{17}$ and $G^{16.2}aU^{16.1}A^{17}$ triplets under MTO, to investigate whether the K_m values were affected (Table 2). The values were nearly identical for all three substrates. This confirms our findings for ribozyme I. The extent of cleavage was also followed for the substrate GfUA (Figure 2). There was 75% cleavage in the first 2 min with a single rate, being identical with the GUA cleavage (data not shown) and with that generally observed for well-behaved ribozymes (*10*). Thus, the lack of the 2′-hydroxy group has no effect on the extent of cleavage.

It is tempting to attribute the high activity for GfU cleavage to the high content, nearly 80%, of the $C_{3'}$-*endo* conformer, which is higher than the population of this conformer present in ribonucleosides, 2′-aminonucleosides, and 2′-deoxynucleosides (*16, 17*). However, the cleavage kinetics do not follow this relationship in a linear manner, and, therefore, factors other than the nucleotide conformation must also govern the catalytic activity.

These data establish that, contrary to previous results, the 2′-hydroxy group at position 16.1 is not essential for activity as established in a variety of sequence contexts. Thus, even though X-ray structure analysis shows H-bonds of this hydroxy group to the N-3 nitrogen of A6 and to the O-4′ oxygen of furanose of C17, they are of no consequence either for the formation of the ground state structure or for the cleavage reaction. Thus, their close proximity in the X-ray structures might be considered as fortuitous and formed only because of the presence of the hydroxy group.

This analysis falls into the more general area of the relationship between X-ray structural and chemical modification data of the hammerhead ribozyme (*12, 13, 18*). Despite considerable agreement between X-ray structure and chemical modifications resulting in modulation of activity, there are a number of discrepancies. Other examples related to the importance of 2′-hydroxy groups include positions G5 and G8 where the arabinosyl derivatives and the 2′-deoxy derivative of G8 are tolerated although the 2′-hydroxy groups are involved in H-bonding interactions. Some of these discrepancies arise from the fact that X-ray structures are ususally of the ground-state conformations, whereas the chemical modification data refer to the transition state. This, however, is not the case for the 2′-hydroxy group at 16.1 where the H-bond is seen in a X-ray structure approaching the transition state (*3*).

In conclusion, the 2′-hydroxy group at position 16.1 has no effect on the hammerhead-catalyzed cleavage of substrates and thus has no functional importance in hammerhead ribozyme catalysis.

ACKNOWLEDGMENT

We thank U. Kutzke for the synthesis of oligonucleotides, W. Pieken (NeXstar Inc.) for a gift of 2′-fluorouridine and 2′-aminouridine phosphoramidites, and S. Verma for careful reading of the manuscript.

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BI991332N