Subscriber access provided by American Chemical Society

Communication

Fast Cleavage Kinetics of a Natural Hammerhead Ribozyme

Marella D. Canny, Fiona M. Jucker, Elizabeth Kellogg,
Anastasia Khvorova, Sumedha D. Jayasena, and Arthur Pardi

J. Am. Chem. Soc., **2004**, 126 (35), 10848-10849• DOI: 10.1021/ja046848v • Publication Date (Web): 14 August 2004

Downloaded from http://pubs.acs.org on April 1, 2009

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 9 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

JACS COMMUNICATIONS

Published on Web 08/14/2004

Fast Cleavage Kinetics of a Natural Hammerhead Ribozyme

Marella D. Canny, Fiona M. Jucker, Elizabeth Kellogg, Anastasia Khvorova,[†] Sumedha D. Jayasena,[‡] and Arthur Pardi*

Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, Colorado 80309-0215

Received May 27, 2004; E-mail: arthur.pardi@colorado.edu

The hammerhead ribozyme is a small catalytic RNA motif, found in plant RNA viruses, satellite RNA, viroids, and repetitive satellite DNA.[1] This ribozyme catalyzes cleavage and ligation of RNA during rolling circle replication.[2] The well-characterized minimal catalytic motif consists of a three-helix junction with an 11-nucleotide conserved core that cleaves a specific phosphodiester bond in a Mg^{2+}-dependent reaction.[3] Crystal structures of this minimal hammerhead ribozyme have a Y-shaped conformation.[4] However, discrepancies between the structural and biochemical data suggest that the conserved core is not in the catalytically competent conformation in the crystal structure.[5]

The minimal hammerhead motif is inactive under physiological conditions and requires high Mg^{2+} for activity. In contrast, larger natural hammerheads are active under physiological conditions and contain motifs outside the conserved core that lower the requirement for Mg^{2+}.[1,6,7] Recent studies have shown that the enhanced catalysis is brought about by a tertiary interaction between hairpin- or internal loops in stems I and II (Figure 1A).[6,7] Previous studies on the minimal hammerhead motif showed that cross-linking of stems I and II increases the ligation rate by over 25-fold.[8] In addition, a recent crystal structure of a minimal hammerhead where stems I and II interact through interstrand stacking has the scissile bond in a conformation susceptible for in-line attack.[9] Thus, there is now strong evidence that proximity of stems I and II is important for stabilizing catalytically competent conformations for both cleavage and ligation.

The natural hammerhead ribozymes are much more efficient catalysts than any previously characterized minimal hammerhead sequences, with rates of cleavage of these natural hammerheads at higher Mg^{2+} becoming too fast to measure manually.[1,6,7] To further probe the full catalytic power of the hammerhead ribozyme, rapid quench flow methods were used here to measure the cleavage rate of a natural hammerhead ribozyme. The satellite DNA of *Schistosoma mansoni* contains a hammerhead ribozyme that has a hairpin loop at the end of stem II and an internal loop in stem I.[10] Single-turnover kinetics were used to characterize the Mg^{2+} and pH dependence of the cleavage reaction of this ribozyme and give cleavage rates of over 870 min^{-1} at 25 °C.

The trans-cleaving construct in Figure 1A is derived from the *Schistosoma* hammerhead. Figure 1C shows that a substrate with the wild-type internal loop on stem I cleaves with a rate of 0.3 min^{-1} under physiological conditions (50 mM Tris pH 7.0, 100 mM NaCl, 100 μM $MgCl_2$). In contrast, a control substrate that has Watson−Crick base pairs in place of the internal loop in stem I (Figure 1B) is not cleaved under these conditions. These results confirm that the interaction between stems I and II is the basis for the difference in cleavage rates of the minimal and the natural hammerhead ribozymes.

[†] Dharmacon, Inc. Lafayette, CO 80026.
[‡] Amgen, Inc., Thousand Oaks, CA 91320.

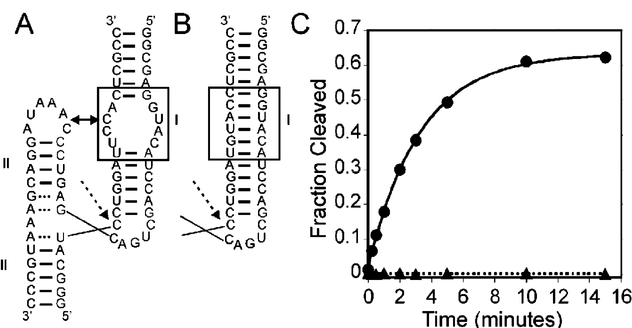

Figure 1. *Schistosoma* hammerhead ribozyme is active under physiological conditions. (A) Sequence of the trans-cleaving ribozyme−substrate construct with the wild-type internal loop motif in stem I (boxed region). A single-headed arrow indicates the cleavage site, and the double-headed arrow indicates the loop−loop tertiary interaction. (B) Control construct where the sequence of the substrate in stem I was changed to a continuous helix instead of an internal loop. (C) Cleavage kinetics for the wild-type (circles) and control (triangles) substrates under physiological conditions (50 mM Tris pH 7.0, 100 mM NaCl, 100 μM $MgCl_2$). The wild-type substrate has a k_{obs} of 0.3 ± 0.1 min^{-1} at 25 °C.

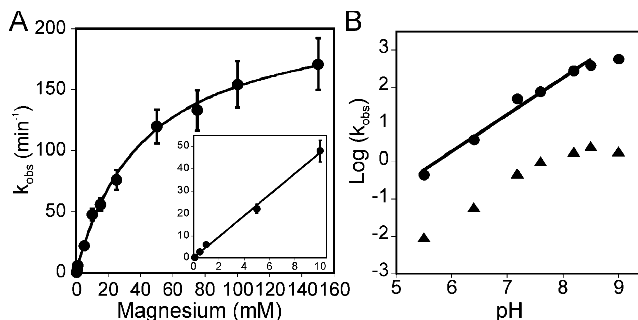

Figure 2. Magnesium and pH dependence of the *Schistosoma* hammerhead catalyzed cleavage. (A) k_{obs} at pH 7.0 as a function of Mg^{2+}. The data fit well to a two-state model ($R = 0.99$) where the k_{obs} plateaus at 220 ± 20 min^{-1}, and 40 ± 6 mM Mg^{2+} is required to obtain half-maximum cleavage rate (see Supporting Information). The inset shows k_{obs} between 100 μM and 10 mM Mg^{2+}. (B) pH dependence of cleavage is shown at 100 μM Mg^{2+} (triangles) and at 10 mM Mg^{2+} (circles) in 50 mM buffer (buffers are listed in Supporting Information). The error bars on the data points are within the symbols. The slopes of log(k_{obs}) with pH are 0.83 ± 0.07 and 0.98 ± 0.07 for 100 μM and 10 mM Mg^{2+}, respectively. The cleavage data were measured to pH 9.0, but this pH was not used in calculating the slopes because multiple groups in the RNA may start to deprotonate at this high pH.

Divalent metal ions are required for efficient cleavage by the hammerhead ribozyme; thus, the cleavage rates of the *Schistosoma* hammerhead construct were measured as a function of Mg^{2+} (Figure 2A). As seen in the inset in Figure 2A, the observed rate of cleavage (k_{obs}) is proportional to Mg^{2+} over a wide range (100 μM to 10 mM) and was fit to a two-state model for Mg^{2+} binding (see Supporting Information). These data show no evidence for cooperativity of Mg^{2+} binding (Hill coefficient = 0.92 ± 0.07) and give

10.1021/ja046848v CCC: $27.50 © 2004 American Chemical Society

a $[Mg^{2+}]_{1/2}$ of ~40 mM (where $[Mg^{2+}]_{1/2}$ is the Mg^{2+} concentration required to achieve half the maximum cleavage rate). Compared to the minimal hammerhead motif, the natural hammerhead ribozyme requires 100-fold less Mg^{2+} to achieve a rate of cleavage of 1.0 min^{-1}, and at pH 7.0, 10 mM Mg^{2+}, this natural hammerhead cleaves ~50-fold faster (48 min^{-1}) than do previously characterized well-behaved minimal hammerheads.[11]

Lilley and co-workers recently used fluorescence resonance energy transfer to show that the trans construct of the *Schistosoma* hammerhead with a single deoxyribose residue at the cleavage site folds into the Y-shaped conformation at low Mg^{2+} concentrations.[12] They found a $[Mg^{2+}]_{1/2}$ of 160 μM (here $[Mg^{2+}]_{1/2}$ is the Mg^{2+} concentration required to achieve half the maximum folding). However, the results in Figure 2A show a $[Mg^{2+}]_{1/2}$ for activity of 40 mM. Thus, the global folding into the Y-shaped conformation is already highly populated at much lower Mg^{2+} than is required for maximum catalysis. These data demonstrate that there are at least two Mg^{2+} involved in the hammerhead, a tightly associating Mg^{2+} involved in folding into the Y-shape and a weaker associating Mg^{2+} required for activity. This weaker associating Mg^{2+} plays a structural and/or catalytic role. Additional data are required to determine the number of Mg^{2+} ions involved and whether Mg^{2+} association is in the catalytic core or the loop−loop motif.

The proposed mechanism for phosphodiester bond cleavage in the hammerhead requires deprotonation of the 2′OH at the cleavage site. The *Schistosoma* hammerhead ribozyme shows a log-linear pH dependence (slope of 0.98) for 10 mM Mg^{2+} (Figure 2B). A similar pH dependence has been previously observed for the minimal hammerhead.[13] For the 100 μM Mg^{2+} data, k_{obs} deviates from log-linear above pH 7.5 (slope of 0.83), suggesting that there is a pH-dependent conformational change which leads to less efficient cleavage at high pH. However, around physiological pH the k_{obs} is log-linear with pH at both high and low Mg^{2+}. This means cleavage is first order in $[OH^-]$ and is consistent with a mechanism where cleavage is limited by the chemical step.

Since the cleavage rate for the *Schistosoma* hammerhead increases with both Mg^{2+} and pH, the catalytic potential of this ribozyme was probed by measuring the cleavage rate at high Mg^{2+} and high pH. At 200 mM Mg^{2+}, pH 8.5, this hammerhead cleaves with a rate of 870 min^{-1} (data not shown). However, it is important to note, that if product release is slow compared to the ligation rate, the observed rate will be reduced by the ligation reaction. Thus, k_{obs} only represents a lower limit for the cleavage rate. Fast kinetics have also recently been reported for several other ribozymes, an extended Varkud satellite ribozyme[14] and a catalytic ribo-switch,[15] and together these studies demonstrate the impressive catalytic rates that can be achieved by RNA.

Recent estimates for speed limits of phosphotransfer reactions in ribozymes predict that the hammerhead ribozyme uses multiple mechanisms to achieve such efficient catalysis.[16] Mechanisms available for RNA catalysis include: stabilization of the scissile bond in a conformation for in-line nucleophilic attack, providing a general base for deprotonating the 2′ hydroxyl group, stabilization of the charge of the 5′ leaving group, or stabilizing the conformation of the transition state through hydrogen bonding and charge stabilization.[17] Although there have been extensive biochemical and structural studies on the minimal hammerhead motif, we still have no clear understanding of the catalytic mechanism of this ribozyme. This is in contrast to several other ribozymes where X-ray structures have rationalized critical aspects of the biochemical/modification data,[18] thus yielding important insights into the catalytic mechanisms. Since the loop−loop interaction in the natural hammerhead helps stabilize the active conformation, some functional groups that were previously found to be critical for activity in the minimal hammerhead may no longer be required in the natural hammerhead. Thus, it will be important to both biochemically and structurally characterize these natural hammerheads. These data may help rationalize the structure−function discrepancies previously observed for the minimal hammerhead.[5]

An interesting question raised by comparing the minimal and natural hammerhead is how the loop−loop interactions far way from the cleavage site can have such a dramatic effect on catalysis. One model is that this new tertiary interaction induces the catalytic conformation at physiological Mg^{2+}, possibly through changes in displacement, twist, or angle of the intervening helical stems. Kinetic studies of other natural hammerhead ribozymes with different loop−loop motifs show similar cleavage rates at 100 μM Mg^{2+} (unpublished results). Thus, even though these natural hammerheads have different loop−loop tertiary interactions, they likely lead to similar conformational changes in the catalytic core.

In summary, kinetic data on the natural *Schistosoma* hammerhead ribozyme show that additional structural motifs outside the catalytic core dramatically enhance catalysis, leading to a cleavage rate of at least 870 min^{-1}. The pH and Mg^{2+} dependence of cleavage is consistent with a model where there is no change in the catalytic mechanism between the minimal and natural ribozyme. Instead, the improved catalysis likely results from increasing the population of conformationally active species. These additional structural elements not only enhance catalysis but are also crucial for use of ribozymes as in vivo cleavage reagents.[6]

Acknowledgment. This work was supported by NIH AI30726.

Supporting Information Available: Description of the experimental methods used for measuring cleavage and fitting the kinetic data. This information is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Forster, A. C.; Jeffries, A. C.; Sheldon, C. C.; Symons, R. H. *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 249−259.
(2) Flores, R.; Hernandez, C.; de la Pena, M.; Vera, A.; Daros, J. A. *Ribonucleases, Pt. A* **2001**, *341*, 540−552.
(3) Hammann, C.; Lilley, D. M. J. *ChemBioChem* **2002**, *3*, 691−700.
(4) Wedekind, J. E.; McKay, D. B. *Annu. Rev. Biophys. Bioeng.* **1998**, *27*, 475−502.
(5) McKay, D. B. *RNA* **1996**, *2*, 395−403.
(6) Khvorova, A.; Lescoute, A.; Westhof, E.; Jayasena, S. D. *Nat. Struct. Biol.* **2003**, *10*, 708−712.
(7) De la Pena, M.; Gago, S.; Flores, R. *EMBO J.* **2003**, *22*, 5561−5570.
(8) Stage-Zimmermann, T. K.; Uhlenbeck, O. C. *Nat. Struct. Biol.* **2001**, *8*, 863−867.
(9) Dunham, C. M.; Murray, J. B.; Scott, W. G. *J. Mol. Biol.* **2003**, *332*, 327−336.
(10) Ferbeyre, G.; Smith, J. M.; Cedergren, R. *Mol. Cell. Biol.* **1998**, *18*, 3880−3888.
(11) Hertel, K. J.; Herschlag, D.; Uhlenbeck, O. C. *Biochemistry* **1994**, *33*, 3374−3385.
(12) Penedo, J. C.; Wilson, T. J.; Jayasena, S. D.; Khvorova, A.; Lilley, D. M. J. *RNA* **2004**, *10*, 880−888.
(13) Clouet, D. O. B.; Uhlenbeck, O. C. *RNA* **1996**, *2*, 483−491.
(14) Zamel, R.; Poon, A.; Jaikaran, D.; Andersen, A.; Olive, J.; De Abreu, D.; Collins, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 1467−1472.
(15) Winkler, W.; Nahvi, A.; Roth, A.; Collins, J.; Breaker, R. *Nature* **2004**, *428*, 263−264.
(16) Breaker, R.; Emilsson, G.; Lazarev, D.; Nakamura, S.; Puskarz, I.; Roth, A.; Sudarsan, N. *RNA* **2003**, *9*, 949−957.
(17) Emilsson, G. M.; Nakamura, S.; Roth, A.; Breaker, R. R. *RNA* **2003**, *9*, 907−918.
(18) Bevilacqua, P. C.; Brown, T. S.; Nakano, S.; Yajima, R. *Biopolymers* **2004**, *73*, 90−109.

JA046848V