

Single-Molecule Visualization of the Zn^{2+} -Dependent DNAzyme Functions

Masayuki Endo,*^[a] Yosuke Takeuchi,^[b] Yuki Suzuki,^[b] Tomoko Emura,^[b] Kumi Hidaka,^[b] Fuan Wang,^[d] Itamar Willner,*^[d] and Hiroshi Sugiyama*^[a] ^[b] ^[c]

Abstract: We demonstrate the single-molecule imaging of the catalytic reaction of the Zn^{2+} -dependent DNAzyme in a DNA origami nanostructure. The single-molecule catalytic activity of a Zn^{2+} -dependent DNAzyme was examined in the designed nanostructure called DNA frame. The DNAzyme and a substrate strands attached to two supported dsDNAs were assembled into the DNA frame in two different configurations. The reaction was monitored by observing the configuration change of the incorporated DNA strands in the DNA frame, which was clearly observed in accordance with the progress of the reaction. The separation of the dsDNAs, induced by the cleavage by the DNAzyme, was directly visualized by high-speed atomic force microscopy (AFM). This nanostructure-based AFM imaging is applicable for monitoring various chemical and biochemical catalytic reactions at the single-molecule level.

Catalytic nucleic acids (DNAzymes) attract growing scientific interest, and different applications of DNAzymes have been discussed.^[1] These include the use of DNAzymes as catalysts that stimulate biocatalytic transformations,^[2] as amplifying labels for sensing events,^[3] as catalysts that transduce logic-gate operations,^[4] as biomolecular switching devices,^[5] as functional units for the controlled release of substrates entrapped in nanocontainers^[6] and more.^[7] One specific class of DNAzymes includes metal-ion-dependent DNAzymes. Different DNA sequences dependent on metal-ion cofactors such as Mg^{2+} , Ca^{2+} , Zn^{2+} , UO_2^{2+} , Hg^{2+} and more, were reported.^{[8][9]} These metal-ion-dependent DNAzymes were applied as amplifying labels for sensing,^[10] as functional components to construct logic gates^[11] and computing circuits, and as stimuli-responsive DNA switches.^[12]

High-speed atomic force microscopy (AFM) measurements were recently implemented to probe DNA nanostructures and their reactivity at the single-molecule level.^[13] In these

experiments, the individual DNA nanostructures were assembled on origami frames, and the reactions of the nanostructures were elucidated by analyzing a collection of frames. For example, DNA structural changes including the G-quadruplex reconfiguration,^[14] the B-Z configurational transitions,^[15] the switchable association and dissociation of photopresponsive DNA,^[16] and visualization of enzymatic processes on DNA nanostructures^[17] were demonstrated. Because the target DNA strands are directly attached to the nano-scaffold, the DNA structural changes can be controlled in the nanospace by arranging the structures inside the DNA nanoscaffold.

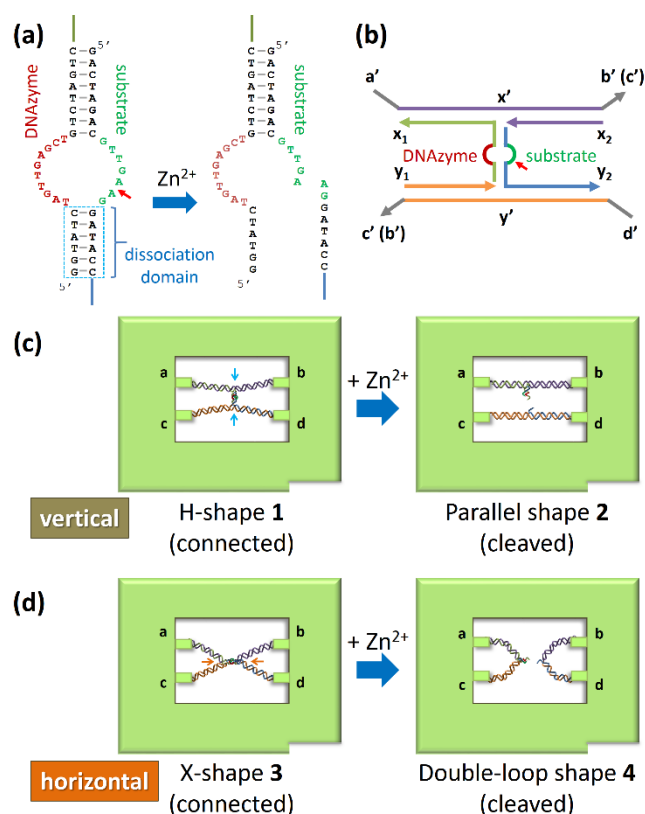


Figure 1. Single-molecule observation system for the DNAzyme mediated cleavage reaction using a DNA frame. (a) DNAzyme and the substrate sequence used in the experiments. Addition of Zn^{2+} induces the cleavage at a specific site (red arrow). The number of base pairs (bp) in the dissociation domain of DNAzyme (dashed light blue rectangle) was varied (4, 6, and 8 bp). (b) Schematic representation of DNA strands incorporated into the DNA frame. (c) Vertical configuration in the DNA frame in the initial state (left; H-shape 1) and the product after the cleavage with Zn^{2+} ion (right; parallel shape 2). (d) Horizontal configuration in the DNA frame in the initial state (left; X-shape 3) and the product after the cleavage with Zn^{2+} ion (right; double-loop shape 4).

- [a] Prof. Dr. M. Endo, Prof. Dr. H. Sugiyama
Institute for Integrated Cell-Material Sciences (WPI-iCeMS)
Kyoto University
Yoshida-ushinomiya-cho, Sakyo-ku, Kyoto 606-8501 (Japan)
E-mail: endo@kuchem.kyoto-u.ac.jp; hs@kuchem.kyoto-u.ac.jp
- [b] Y. Takeuchi, Dr. Y. Suzuki, T. Emura, K. Hidaka, Prof. Dr. H. Sugiyama
Department of Chemistry, Graduate School of Science
Kyoto University
Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502 (Japan)
- [c] Prof. Dr. M. Endo, Prof. Dr. H. Sugiyama
CREST
Japan Science and Technology Agency (JST)
Sanbancho, Chiyoda-ku, Tokyo 102-0075 (Japan)
- [d] Dr. F. Wang, Prof. Dr. I. Willner
Institute of Chemistry, The Minerva Center for Biohybrid Complex Systems
The Hebrew University of Jerusalem
Jerusalem 91904 (Israel)
E-mail: willner@vms.huji.ac.il

Supporting information for this article is given via a link at the end of the document.

In the present study, we applied the DNA nanospace to follow the catalytic DNA, Zn^{2+} -dependent DNAzyme, at the single-molecule level. For observation of the substrate cleavage by the DNAzyme, we introduced the DNAzyme and substrate between the two double-stranded DNAs (dsDNAs) in a DNA

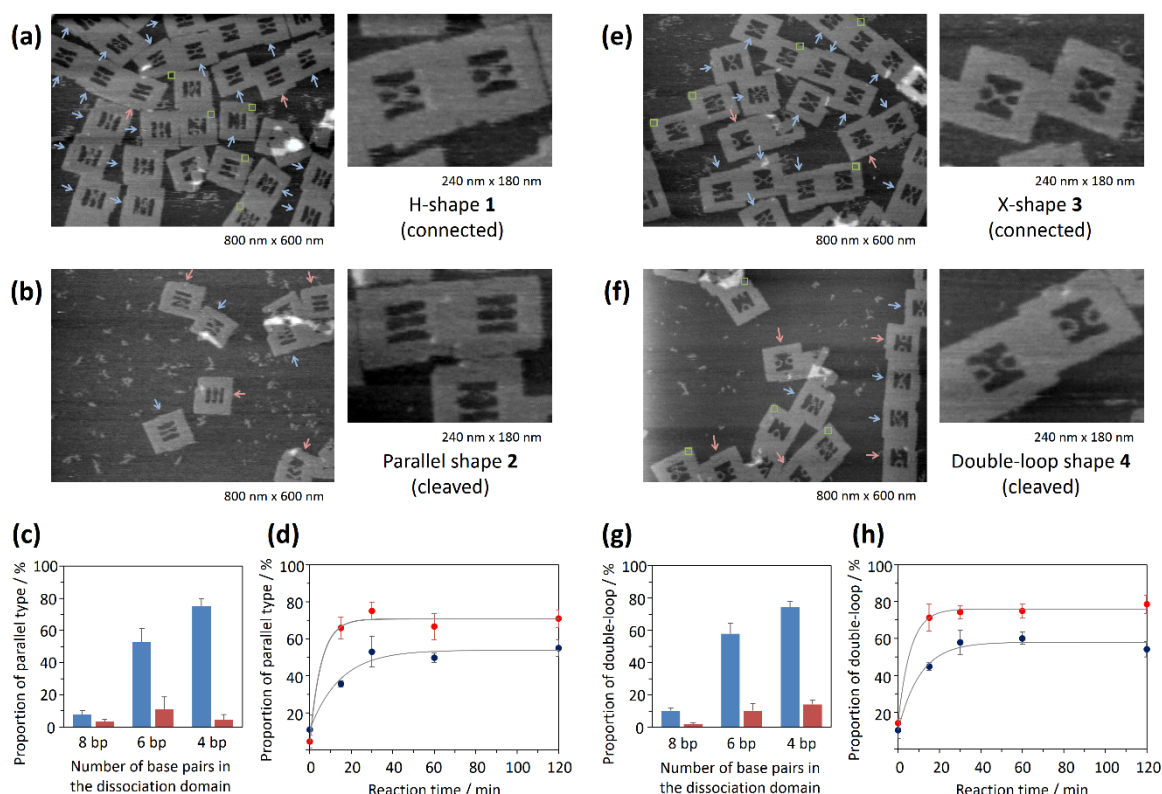


Figure 2. AFM images corresponding to the Zn^{2+} -dependent DNAzyme catalyzed cleavage of the substrate DNA in the DNA frame. (a) AFM images of DNA frames in the vertical configuration in the initial state. Blue and red arrows indicate the DNA frame containing H-shape (1) and parallel-shape (2) dsDNAs, respectively. The green rectangle represents an unclear DNA frame. (b) AFM images of DNA frames in the vertical configuration after incubation with ZnCl_2 . (c) The summary of the substrate cleavage by a DNAzyme with 4, 8, and 6 bp dissociation domain in the vertical configuration. Red and blue bars represent the parallel shape formation in the initial and after the incubation with Zn^{2+} at 30 °C for 30 min, respectively. (d) Time-dependent parallel shape formation by DNAzyme with 4 bp (red circle) and 6 bp (blue circle) dissociation domain. Error bars represent mean $\pm$ S.D. obtained from the three independent experiments. (e) AFM images of DNA frames in a horizontal configuration in the initial state. Blue and red arrows indicate the DNA frame containing an X-shape (3) and double-loop-shaped (4) dsDNAs, respectively. (f) AFM images of DNA frames in the horizontal configuration after incubation with Zn^{2+} . (g) The substrate cleavage by a DNAzyme with 4, 8, and 6 bp dissociation domain in the vertical configuration. Red and blue bars represent the parallel shape formation in the initial and after the incubation, respectively. (h) Time-dependent parallel shape formation by DNAzyme with 4 bp (red circle) and 6 bp (blue circle) dissociation domain.

frame (Figure 1).^[17] The DNAzyme we employed is the Zn^{2+} -ion-dependent DNAzyme that selectively cleaves the substrate DNA at a sequence-specific site (Figure 1).^[18] Here, we connected the DNAzyme to one supported dsDNA and the substrate to another, and subsequently incorporated them into the DNA frame, using the four single-stranded DNA tethers as linkers to the DNA origami frame (Figure 1b). We generated two different configurations of the DNAzyme sequence/substrate pair for the insertion into the DNA frame. In the vertical configuration of DNAzyme/substrate dsDNA in the DNA frame, the two supported dsDNAs were connected to the a-b and c-d sites of the DNA frame (1; Figure 1c). In the horizontal configuration, the two dsDNAs were connected to the a-c and b-d sites (3; Figure 1d).

First, we examined the substrate DNA cleavage using a DNAzyme oligonucleotide (Figures S1, S2). We conducted the reaction in a solution containing 10 mM Tris-buffer (pH 7.0), 20 mM MgCl_2 , and metal ion at 30 °C for 30 min. The cleavage selectively occurred in the presence of Zn^{2+} , and no cleavage was observed in the presence of other ions such as Cu^{2+} or Ni^{2+} as reported previously (Figure S1a).^[18] Then we examined the reaction in the DNA nanostructure using the same reaction conditions. The DNAzyme and substrate DNA supported by two dsDNAs were introduced into a DNA frame by pre-annealing the DNA components in a solution containing 10 mM Tris-buffer (pH 7.0) and 20 mM MgCl_2 (Figure S3). After the assembly, the structures were observed by AFM. The two supported dsDNAs

were connected to the DNA frame in >90% yield (Figure 2). A center DNAzyme/substrate dsDNA in the vertical configuration was clearly identified, and the intact structure was observed as an H-shaped construct (Figures 2a and S4). In the case of the horizontal configuration, the DNAzyme and substrate DNA supported by two dsDNAs were assembled into the DNA frame (Figure S9). The center dsDNA was also clearly identified, and the whole structure was observed as an X-shape (Figures 2d and S10). The DNAzyme/substrate dsDNA in the vertical and horizontal configurations could be clearly visualized between the two supported dsDNAs in the DNA frame (Figure 2a,e).

Next, we examined the cleavage reaction of the substrate DNA in the DNA frame. The reaction was performed in the presence of 2 mM ZnCl_2 at 30 °C for 30 min. After incubation with Zn^{2+} in the DNA frame, the separation of the central part of the DNAzyme/substrate dsDNA was clearly observed (Figure 2). In the case of the vertical configuration, we observe the separation of two supported dsDNAs to be in a parallel shape (Figures 2b and S5). We compared the effect on the separation by changing the number of base pairs in the dissociation domain with 4, 6, and 8 base pairs (bp) (Figures 2c, S6, S7, S8). After incubation with Zn^{2+} , the parallel shape increased in the order of 4, 6, 8 bp, and the observed separation in the 8 bp dissociation domain was modest. These results indicate that the number of base pairs in the dissociation domain affects the separation in the DNA frame after the cleavage. We examined the time-dependent formation of the parallel shape in the DNA frame

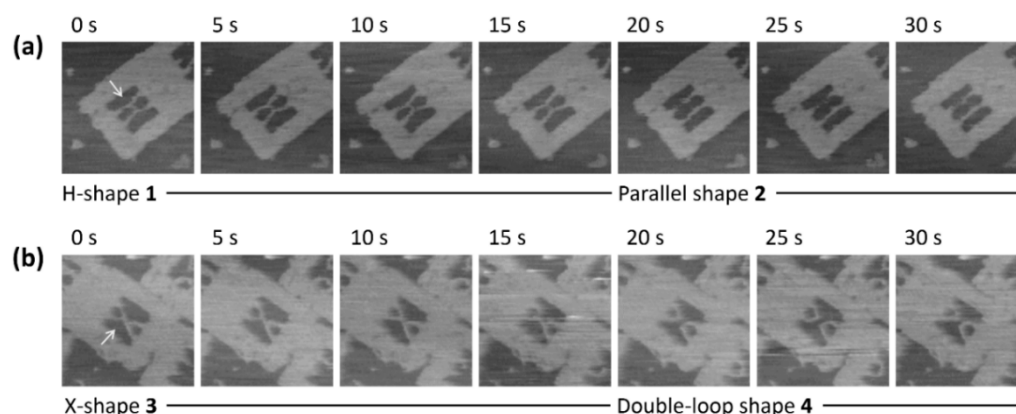


Figure 3. Time-lapse AFM images for the dissociation of DNA strands induced by the cleavage of substrate DNA by the DNAzyme in the DNA frame. (a) Lapsed images of the reaction in the vertical-type configuration in the presence of ZnCl_2 . (b) Lapsed images of the reaction in the double-loop configuration in the presence of ZnCl_2 . Time 0 s is an arbitrary time during the AFM scanning. Scanning rate, 0.2 frame/s. Image size 150 nm x 150 nm.

using DNAzymes with 4 and 6 bp dissociation domain (Figures 2d, S7, S8). In both cases, the reactions were saturated around 30 min, and the yield of the parallel shape formation using DNAzyme with 4 bp dissociation domain was higher than that with 6 bp. The observed rate constants for the parallel shape formation by DNAzymes with 4 bp and 6 bp were 0.18 min^{-1} and 0.068 min^{-1} , respectively, which indicates that the dissociation step directly affects the kinetics of the parallel shape formation. In the cases of the horizontal configuration, we also observed the separation of two supported dsDNAs that turn upon cleavage into a double-loop shape (Figures 2f and S11). We also observed the effect of the dissociation domain on the double-loop formation. The tendency was similar to the vertical configuration (Figures 2g, S12, S13, S14). The observed rate constants for the double-loop formation with 4 bp and 6 bp dissociation domain were 0.17 min^{-1} and 0.094 min^{-1} , respectively, which also showed the similar tendency to the vertical configuration (Figure 2h). The cleavage reaction in an ensemble state showed comparable rate constant (0.48 min^{-1} ; Figure S1b), considering the additional dissociation step of the cleaved substrate and DNAzyme in the DNA frame. These results indicate that the DNAzyme mediated reaction in the DNA frame reflects the reaction in the ensemble state.

In the two configurations, the yields of the reactions were slightly higher in the horizontal configuration than those in the vertical configuration. The lengths of the DNAzyme/substrate dsDNA were different in two configurations, which should affect the subsequent dissociation. We directly measured the lengths of the DNAzyme/substrate dsDNA in the vertical and horizontal configurations in the AFM images, and the distances between the two supported dsDNAs at the center in these configurations were $13.5 \pm 1.8 \text{ nm}$ and $10.7 \pm 2.0 \text{ nm}$, respectively (Figures 2a,e, and S15). The tension imposed on the center dsDNA in the vertical configuration should be larger than that in the horizontal configuration. The results may suggest that the active structure of the catalytic domain was maintained in both configurations even the DNAzyme/substrate was expanded by the two supported strands.

Finally, we examined the imaging of the catalytic activity of the DNAzyme at the single-molecule level using a high-speed AFM. We prepared a sample in the absence of Zn^{2+} and observed the sample by high-speed AFM in the presence of Zn^{2+} . The prepared sample was adsorbed on mica, and then the sample was observed in the observation buffer containing Zn^{2+} .

First, we examined the vertical configuration. We find that the H-shaped structure in the vertical configuration changed to the separated dsDNAs during AFM scanning (arrow in Figures 3a and S16a). The separation of the dsDNA occurred and the corresponding conversion of the H-shaped structure into the two separated parallel dsDNAs was clearly observed at time 20 s (Figure 3a). This observation suggests that a cleavage reaction followed by separation of dsDNAs occurred. In the case of the horizontal configuration, we also observed the structural conversion from an X-shaped structure to two separated loops at time 20 s (arrow in Figures 3b and S16b). These results suggest that the single-molecule cleavage by the DNAzyme and subsequent separation can be monitored using this observation system and high-speed AFM imaging.

In conclusion, we have demonstrated the single-molecule imaging of the cleavage reaction and dissociation of dsDNA by a Zn^{2+} -dependent DNAzyme occurring in a DNA nanostructure. The reaction can be clearly monitored by observing the configuration change of the dsDNAs incorporated into the DNA frame. Using high-speed AFM, dynamic movement of the DNA strands induced by the cleavage with the DNAzyme was visualized in the DNA frame. This versatile observation system described here can be a novel approach for monitoring the target chemical and biochemical reactions that are designed to induce structural arrangements of the multiple DNA strands in the defined DNA nanospace.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Molecular Robotics" (Grant Number 24104002) of MEXT, Core Research for Evolutional Science and Technology (CREST) of JST, and JSPS KAKENHI (Grant Numbers 15H03837, 25253004, 26620133). IW is supported by the Israel Science Foundation. Financial supports from the Sekisui Chemical Research Grant and the Kurata Memorial Hitachi Science and Technology Foundation to ME are also acknowledged.

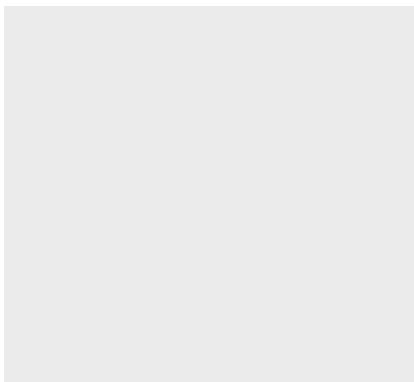
Keywords: DNAzyme • DNA origami • single-molecule imaging • high-speed atomic force microscopy • structural change

[1] a) R. R. Breaker, G. F. Joyce, *Chem. Biol.*, **1994**, *1*, 223–229; b) I. Willner, B. Shlyahovsky, M. Zayats, B. Willner, *Chem. Soc. Rev.*, **2008**, *37*,

- 1153–1165; c) M. Famulok, J. S. Hartig, G. Mayer, *Chem. Rev.*, **2007**, *107*, 3715–3743; d) G. F. Joyce, *Angew. Chem., Int. Ed.*, **2007**, *46*, 6420–6436; e) J. Andreasson, S. D. Straight, S. Bandyopadhyay, R. H. Mitchell, T. A. Moore, A. L. Moore, D. Gust, *J. Phys. Chem. C*, **2007**, *111*, 14274–14278.
- [2] a) E. Golub, R. Freeman, I. Willner, *Angew. Chem. Int. Ed.*, **2011**, *50*, 11710–11714. (b) Z.-G. Wang, P. Zhan, B. Ding, *ACS Nano*, **2013**, *7*, 1591–1598. (c) M. Wilking, U. Hennecke, *Org. Biomol. Chem.*, **2013**, *11*, 6940–6945.
- [3] a) R. Freeman, J. Girsh, I. Willner, *ACS Appl. Mater. Interfaces*, **2013**, *5*, 2815–2834; b) Y. Du, B. Li, E. Wang, *Acc. Chem. Res.*, **2013**, *46*, 203–213; c) Y. V. Gerasimova, D. M. Kolpashchikov, *Chem. Biol.*, **2010**, *17*, 104–106; d) G. Pelossof, R. Tel-Vered, I. Willner, *Anal. Chem.*, **2012**, *84*, 3703–3709; e) R. Orbach, F. Wang, O. Lioubashevsky, R. D. Levine, F. Remacle, I. Willner, *Chem. Sci.*, **2014**, *5*, 3381–3387; f) F. Wang, C.-H. Lu, I. Willner, *Chem. Rev.*, **2014**, *114*, 2881–2941.
- [4] a) S. Shimron, N. Magen, J. Elbaz, I. Willner, *Chem. Commun.*, **2011**, *47*, 8787–8789; b) C. Teller, S. Shimron, I. Willner, *Anal. Chem.*, **2009**, *81*, 9114–9119; c) J. Elbaz, S. Shimron, I. Willner, *Chem. Commun.*, **2010**, *46*, 1209–1211; d) G. Seelig, D. Soloveichik, D. Y. Zhang, E. Winfree, *Science*, **2006**, *314*, 1585–1588; e) A. Padirac, T. Fujii, Y. Rondelez, *Curr. Opin. Biotechnol.*, **2013**, *24*, 575–580; f) L. Qian, E. Winfree, *Science*, **2011**, *332*, 1196–1201; g) L. Qian, E. Winfree, J. Bruck, *Nature*, **2011**, *475*, 368–372; h) R. R. Breaker, *Nat. Biotechnol.*, **1997**, *15*, 427–431.
- [5] a) B. Ge, Y. C. Huang, D. Sen, H. Z. Yu, *Angew. Chem., Int. Ed.*, **2010**, *49*, 9965–9967. (b) X. Liu, A. Niazov-Elkan, F. Wang, I. Willner, *Nano Lett.*, **2013**, *13*, 219–225. (c) S. Shimron, N. Magen, J. Elbaz, I. Willner, *Chem. Commun.*, **2011**, *47*, 8787–8789.
- [6] M. Moshe, J. Elbaz, I. Willner, *Nano Lett.*, **2009**, *9*, 1196–1200.
- [7] C. H. Lu, X. J. Qi, R. Orbach, H.-H. Yang, I. Mironi-Harpaz, D. Seliktar, I. Willner, *Nano Lett.*, **2013**, *13*, 1298–1302.
- [8] a) R. R. Breaker, G. F. Joyce, *Trends Biotechnol.*, **1994**, *12*, 268–275; b) J. Tang, R. R. Breaker, *Proc. Natl. Acad. Sci. U.S.A.*, **2000**, *97*, 5784–5789.
- [9] a) J. W. Liu, A. K. Brown, X. L. Meng, D. M. Cropek, J. D. Istok, D. B. Watson, Y. Lu, *Proc. Natl. Acad. Sci. U.S.A.*, **2007**, *104*, 2056–2061; b) J. Liu, Y. Lu, *J. Am. Chem. Soc.*, **2007**, *129*, 9838–9839.
- [10] a) C. H. Fan, K. W. Plaxco, A. J. Heeger, *Proc. Natl. Acad. Sci. U. S. A.*, **2003**, *100*, 9134–9137. (b) F. Wang, J. Elbaz, R. Orbach, N. Magen, I. Willner, *J. Am. Chem. Soc.*, **2011**, *133*, 17149–17151.
- [11] a) M. N. Stojanovic, T. E. Mitchel, D. Stefanovic, *J. Am. Chem. Soc.*, **2002**, *124*, 3555–3561; b) M. N. Stojanovic, D. Stefanovic, *Nat. Biotechnol.*, **2003**, *21*, 1069–1074; c) L. Wang, J. Zhu, L. Han, L. Jin, C. Zhu, E. Wang, S. Dong, *ACS Nano*, **2012**, *6*, 6659–6666; d) R. Orbach, L. Mostinski, F. Wang, I. Willner, *Chem. Eur. J.*, **2012**, *18*, 14689–14694; e) R. Orbach, F. Remacle, R. D. Levine, I. Willner, *Proc. Natl. Acad. Sci. U.S.A.*, **2012**, *109*, 21228–21233; f) J. M. Picuri, B. M. Frezza, M. R. Ghadiri, *J. Am. Chem. Soc.*, **2009**, *131*, 9368–9377; g) N. C. Gianneschi, M. R. Ghadiri, *Angew. Chem., Int. Ed.*, **2007**, *46*, 3955–3958; h) Y. Benenson, T. Paz-Elizur, R. Adar, E. Keinan, Z. Livneh, E. Shapiro, *Nature*, **2001**, *414*, 430–434; i) Y. Benenson, B. Gil, U. Ben-Dor, R. Adar, E. Shapiro, *Nature*, **2004**, *429*, 423–429; j) R. Adar, Y. Benenson, G. Linshiz, A. Rosner, N. Tishby, E. Shapiro, *Proc. Natl. Acad. Sci. U.S.A.*, **2004**, *101*, 9960–9965; k) H. Lederman, J. Macdonald, D. Stefanovic, M. N. Stojanovic, *Biochemistry*, **2006**, *45*, 1194–1199.
- [12] a) J. Elbaz, S. Shimron, I. Willner, *Chem. Commun.*, **2010**, *46*, 1209–1211; b) M. Zhou, X. Liang, T. Mochizuki, H. Asanuma, *Angew. Chem., Int. Ed.*, **2010**, *49*, 2167–2170.
- [13] a) M. Endo, H. Sugiyama, *Acc. Chem. Res.*, **2014**, *47*, 1645–1653; b) A. Rajendran, M. Endo, H. Sugiyama, *Chem. Rev.*, **2014**, *114*, 1493–1520; c) A. Rajendran, M. Endo, H. Sugiyama, *Angew. Chem. Int. Ed.*, **2012**, *51*, 874–890.
- [14] a) Y. Sannohe, M. Endo, Y. Katsuda, K. Hidaka, H. Sugiyama, *J. Am. Chem. Soc.*, **2010**, *132*, 16311–16313; b) A. Rajendran, M. Endo, K. Hidaka, H. Sugiyama, *Angew. Chem. Int. Ed.*, **2014**, *126*, 4191–4196.
- [15] a) A. Rajendran, M. Endo, K. Hidaka, H. Sugiyama, *J. Am. Chem. Soc.*, **2013**, *135*, 1117–1123; b) M. Endo, M. Inoue, Y. Suzuki, C. Masui, H. Morinaga, K. Hidaka, H. Sugiyama, *Chem. Eur. J.*, **2013**, *19*, 16887–16890.
- [16] M. Endo, Y. Yang, Y. Suzuki, K. Hidaka, H. Sugiyama, *Angew. Chem. Int. Ed.*, **2012**, *51*, 10518–10522.
- [17] a) M. Endo, Y. Katsuda, K. Hidaka, H. Sugiyama, *J. Am. Chem. Soc.*, **2010**, *132*, 1592–1597; b) M. Endo, Y. Katsuda, K. Hidaka, H. Sugiyama, *Angew. Chem. Int. Ed.*, **2010**, *49*, 9412–9416; c) Y. Suzuki, M. Endo, Y. Katsuda, K. Ou, K. Hidaka, H. Sugiyama, *J. Am. Chem. Soc.*, **2014**, *136*, 211–218.
- [18] H. Gu, K. Furukawa, Z. Weinberg, D. F. Berenson, R. R. Breaker, *J. Am. Chem. Soc.*, **2013**, *135*, 9121–9129.

Entry for the Table of Contents (Please choose one layout)**COMMUNICATION**

The single-molecule catalytic activity of a Zn^{2+} -dependent DNAzyme was investigated in a DNA origami nanostructure. The DNAzyme and substrate strand attached to dsDNAs were assembled into the DNA frame in different configurations. The reaction progress was directly visualized by observing the structural changes of the incorporated DNA strands using high-speed atomic force microscopy (AFM).



Masayuki Endo,* Yosuke Takeuchi, Yuki Suzuki, Tomoko Emura, Kumi Hidaka, Fuan Wang, Itamar Willner,* and Hiroshi Sugiyama*†

Page No. – Page No.

Single-Molecule Visualization of the Zn^{2+} -Dependent DNAzyme Functions