J|A|C|S COMMUNICATIONS

Published on Web 07/27/2010

Pulling Genetic RNA out of Tobacco Mosaic Virus Using Single-Molecule Force Spectroscopy

Ningning Liu,[†] Bo Peng,[‡] Yuan Lin,[‡,§] Zhaohui Su,[‡] Zhongwei Niu,[§] Qian Wang,[§] Wenke Zhang,*[,†] Hongbin Li,[||] and Jiacong Shen[†]

State Key Laboratory of Supramolecular Structure and Materials, College of Chemistry, Jilin University, 2699 Qianjin Street, Changchun 130012, P. R. China, State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, P. R. China, Department of Chemistry and Biochemistry and NanoCenter, University of South Carolina, 631 Sumter Street, Columbia, South Carolina 29208, and Department of Chemistry, University of British Columbia, Vancouver, BC, Canada V6T 1Z1

Received June 16, 2010; E-mail: zhangwk@jlu.edu.cn

Abstract: RNA−coat protein interactions in intact tobacco mosaic virus have been investigated for the first time directly on the single-molecule level by pulling the genetic RNA step by step out of the helical groove formed by its protein coat. The effects of pulling speed and pH on RNA−protein interactions are presented. In addition, the rebinding behavior of the detached RNA with the protein coat is discussed. Our results demonstrate the possibility of studying nucleic acid−protein interactions in more complicated systems using AFM-based single-molecule force spectroscopy.

The interactions between nucleic acids and proteins play a pivotal role in every aspect of molecular biology and cell biology. A good understanding of the mechanism of nucleic acid−protein interactions will help us gain control of many important biological processes, such as virus infection and cancer cell growth. Tobacco mosaic virus (TMV) was the first virus to be discovered, and it has been used as a model system for virus study since its discovery over a century ago.[1] TMV is a rod-shaped particle with a single strand of RNA (∼6390 bases) wrapped inside the helical groove (8 nm diameter) formed by a protein coat that is composed of ∼2130 copies of identical coat proteins (Figure 1a,b).[2−5] Although the static structure of TMV has been known for years, the affinity or strength of interaction between the RNA genome and the coat protein in an intact virus particle is unknown. In addition, the mechanism of virion disassembly during infection still lacks direct experimental support.[7] A good understanding of the RNA−protein interactions and the effect of RNA binding on the stability, assembly, and disassembly of TMV particles will facilitate a deep and direct exploration of the mechanism of virus infection as well as the rational design of novel nanomaterials using TMV as a template.

Atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) is a powerful technique in the investigation of inter- or intramolecular interactions at the single-molecule level, such as antigen−antibody, ligand−receptor, and $\pi-\pi$ interactions; single-molecule motors; protein unfolding; DNA unwinding; and DNA−protein interactions.[8−19] One of the future challenging directions is the study of molecular interactions in more complicated systems. Here we used AFM-based SMFS to study the RNA−coat protein interactions in aqueous solution by pulling the RNA genome out of a TMV particle that was immobilized on a gold substrate, as shown in Figure 1c. The present study extends the force spectroscopy technique to the study of nucleic acid−protein interactions in more complicated biological systems (i.e., in the virus particle).

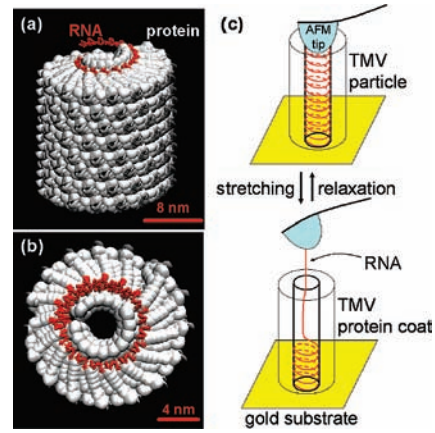

Figure 1. (a) Structure of TMV ($\sim^1/_{17}$ of the total length), (b) its cross section, and (c) schematic illustration of the SMFS experiment on RNA stretching. The TMV structure was created using VMD software[6] together with structural data of TMV from the RCSB Protein Data Bank (PDB entry 2tmv). RNA genome is embedded in the helical groove formed by its protein coat. Every three nucleotides bind to one coat protein. For the sake of clarity, the top five protein subunits at the 5′ end were removed to expose part of the RNA fragments. The cross-section diameters for the TMV particle and the inner channel are 18 and 4 nm, respectively.

Following a literature protocol, the cysteine-added TMV particles (named as TMV1cys; see the Experimental Details in the Supporting Information) were first perpendicularly immobilized on a gold substrate, likely by their 3′ ends, via gold−thiol chemistry.[20] Next, the AFM tip was brought into contact with the immobilized TMV particles. As a result, we hypothesize that the AFM tip was in contact with the 5′ end of the TMV particle. As shown in Figure 1c, the viral RNA could stick to the AFM tip and subsequently be pulled out of the TMV particle upon retraction of the AFM tip, resulting in the observed force−extension curves, which are characterized by sawtooth-like plateaus. The height of each plateau is centered at ∼400 pN (at a pulling speed of 2 μm/s) and fluctuates by approximately ±50 pN, forming a sawtooth-like pattern, as shown in Figures 2 and 3. We attribute such sawtooth-like plateaus to the unwinding and detachment of the RNA from its protein coat (Figure 1).

To further test the origin of the sawtooth-like plateau, we performed a stretching−relaxation experiment on the same TMV particle. The molecule was first stretched to a certain length (∼1000 nm) and then relaxed before it detached from the AFM tip.

[†] Jilin University.
[‡] Changchun Institute of Applied Chemistry.
[§] University of South Carolina.
[||] University of British Columbia.

10.1021/ja1052544

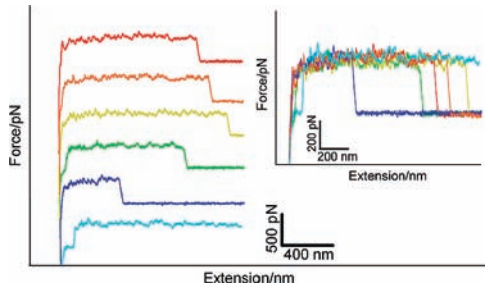

Figure 2. Typical sawtooth-plateau-containing force−extension curves obtained at a pulling speed of 2 μm/s. The force first increases monotonically with extension and then remains relatively constant at ∼400 pN with sawtooth-like fluctuations. The inset shows a superposition of the curves.

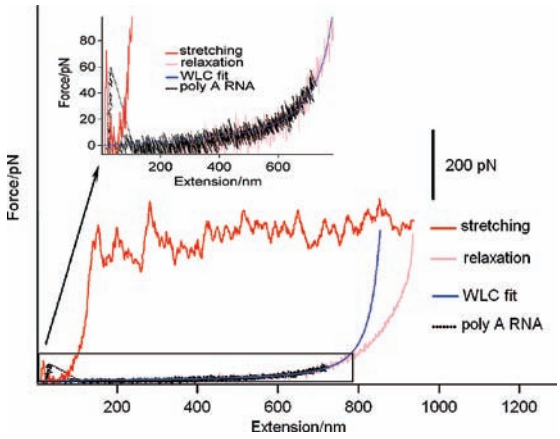

Figure 3. Typical stretching and relaxation curves superimposed with a WLC fit (blue solid curve) and a stretching curve of poly(A) RNA (black dotted curve). The inset shows an enlarged version of the superimposition region below 100 pN.

Figure 3 presents a typical stretching and relaxation curve showing that during the relaxation process, the sawtooth-like plateau disappears and a smooth curve, whose shape corresponds to the elastic behavior of a flexible polymer chain, appears instead. We attribute this elastic behavior to the relaxation of the RNA molecule being pulled out of TMV particle. The following two facts can further support this assumption: first, a worm-like chain (WLC)[19d] fit to the relaxation curve produced a persistence length of ∼0.6 nm, which is very close to that of single-stranded DNA;[16,21] second, a poly(A) RNA stretching curve can be superimposed on it very well. On the basis of these facts, we can draw a conclusion that the plateau in the stretching curve corresponds to the unwinding and detachment of the RNA from its protein coat by the external force. With a control experiment in the presence of RNase, a sudden drop of force from ∼115 to 0 pN was frequently observed on the relaxing curves during the stretching/relaxation cycles (see the upper black curve in Figure S1). In addition, the RNA pulling signal disappeared during the subsequent approach−retract cycle (see the bottom pair of curves in Figure S1). The sudden drop in force may be attributed to the RNA chain cutoff by RNase.

It has been shown that the nonspecific interactions between an AFM tip and a polymer fragment can be 1 nN or higher.[16,19d] This force is sufficient to break most of the inter- (or intra-) molecular interactions, such as the folded protein[15,18] and the RNA−protein interactions in our system. In the stretching experiments at different pulling speeds, we found that the height of the sawtooth-like plateaus increased from 60 pN at a pulling speed of 0.03 μm/s to 400 pN at a pulling speed of 2.5 μm/s (see Figure S2; more systematic studies are underway). The stretching-speed dependence of the unbinding force between RNA and its protein coat indicates that the unbinding process is a nonequilibrium event on the time scale of the SMFS experiment. At a pulling speed of 30 nm/s (a speed close to that of RNA replication under optimized conditions), an average plateau force of ∼60 pN was observed (see the bottom curve in Figure S2). The energy for breaking the binding between a coat protein subunit and three nucleotides can then be roughly estimated as $30.6k_BT$ (or 73.68 kJ/mol, as $k_BT = 4$ pN nm at room temperature), assuming a distance of 0.68 nm between adjacent nucleotides. To our knowledge, this is the first investigation to provide quantitative information on RNA−protein interactions in an intact TMV.

The sawtooth pattern on the plateau can be ascribed to the sequence-dependent unbinding of the RNA from the TMV protein coat. Earlier studies have shown that the trinucleoside diphosphate (TNDP) AAG binds to TMV protein with a relatively greater binding constant (12×10^3 M^{-1}) than other sequences.[22] To further test our hypothesis, statistical analysis was performed on the width distribution of the adjacent sawtooth-like peaks. For comparison, we also analyzed the calculated distances between adjacent AAG TNDPs (as well as several randomly chosen TNDPs; see Figure S3) in the RNA genome of TMV. If the sawtooth-like plateaus are produced by the unwinding and detaching of RNA from its protein coat, then the distribution of the gaps between adjacent peaks in the stretching curves and the distribution of the calculated distances between adjacent AAG sequences should be similar, since the detachment of AAG from the coat proteins should give a stronger unbinding force than other types of TNDP. Our results show that the overall shapes of these two histograms are indeed similar and thus indicative of the sequence-dependent unbinding of RNA from TMV coat protein, as shown in Figure 4.

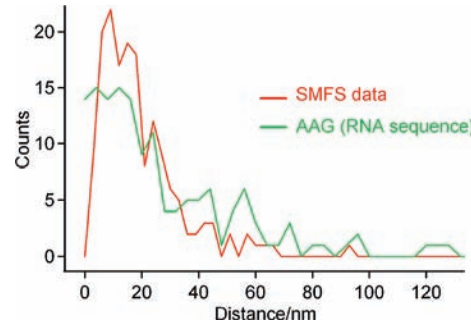

Figure 4. Distribution of the width between adjacent sawtooth-like peaks in the stretching curves (red trace) and distribution of the calculated distance between adjacent AAGs in the TMV RNA genome sequences (green trace).

From the data obtained to this point, we know that it is possible to pull the RNA out of its protein coat using AFM. In doing this, a direct quantitative measurement of RNA−protein interactions can be obtained. This finding supports/extends the idea suggested by the Samori group.[9b] That is, in our current study, we have been able to investigate the multivalent nucleotide−protein interactions on a three-dimensional surface (the helical groove formed by the coat proteins) by using polynucleotide (RNA) as a molecular handle. However, two interesting related questions arise: (1) is the protein coat still there after the RNA is partially pulled out and (2) can the detached RNA fragments find their way back to the helical groove formed by the coat proteins? To answer these two questions, we performed the stretching and relaxation experiment in series on the same molecule. If the protein coat is still there and RNA fragments can find their way back during relaxation, then we should be able to observe the reappearance of the sawtooth plateau on the stretching traces during the subsequent pulling experi-

ment. As shown in Figure 5, the sawtooth pattern is initially observed in the second approach−retraction cycle, and then most of the plateau reappears in the fourth and 15th cycles. Between these cycles, most of the stretching and relaxation curves are quite smooth, and there is little deviation (hysteresis) between them in the long-extension region (see the rightmost parts of the curves in cycles 3, 14, and 19 in Figure 5). Occasionally, such as during the 12th cycle, a partial plateau appears in long-extension area. These results indicate that during the relaxation it is possible for RNA fragments that have been detached from the protein coat to rebind onto the protein coat. In addition, it seems that the detached RNA fragments located in the deeper part of the TMV particle (i.e., RNA fragments that are located away from the end picked up by the AFM tip) find their way back first, since the plateau reappears first from those long-extension areas (see the stretching curves in cycles 4, 12, 15, and 32). In view of the fact that in an intact TMV particle the RNA is embedded in the helical groove formed by its protein coat and is not exposed directly to the inner channel (see Figure 1a,b), it is difficult for the detached RNA fragments (which are located mainly in the inner channel of the TMV particle) to access the helical groove. However, during the stretching−relaxation cycle the RNA molecule is partially pulled out of the TMV particle, and there should still be some intact RNA−protein complexes existing away from the open end out of which the RNA fragment is pulled (see Figure 1c). As a result, such intact structures could serve as "nucleation centers" and trigger the continuous reassembly of the detached RNA fragments with their respective coat proteins from the inside to the opening of the TMV particle in a fashion similar to that of the TMV assembly process, during which the RNA is pulled through the central hole of the growing virus to reach its binding site.[3b] The presence of intact RNA−protein complexes was further confirmed by a control experiment (see Figure S4).

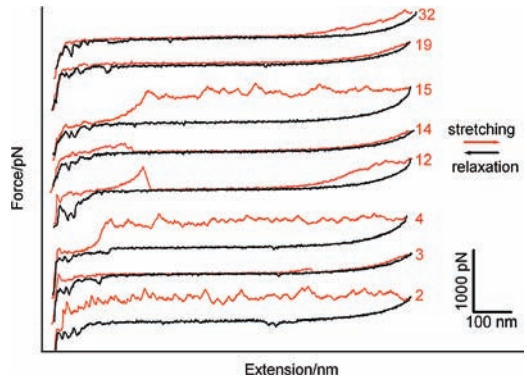

Figure 5. Repeated stretching and relaxation of the same RNA molecule without rupture. The order of the stretching−relaxation cycle is marked numerically.

During our experiment, we also found that results similar to those discussed above could also be obtained on wild-type TMV particles that were immobilized on a gold substrate by physical adsorption (see Figure S5). Furthermore, other factors, such as the solution pH, also affected the RNA−protein interactions (see Figure S6). In addition to the sawtooth-plateau-containing curves, stretching traces with single sharp peaks, which correspond to the stretching of the intact TMV particle, could frequently be observed during our experiment (see Figure S7).

In summary, we have investigated for the first time the RNA−coat protein interactions in an intact TMV directly at the single-molecule level by pulling the RNA genome step by step out of a cylindrical nanotube formed by its coat proteins. In doing this, we have demonstrated the possibility of using AFM-based SMFS to investigate the nucleic acid−protein interactions in a more complicated system. SMFS's high force sensitivity and friendly working platform (e.g., can work in aqueous solution) allow the effects of other factors (e.g., pH, ionic strength, RNA binding reagents) on RNA−protein interactions to be systematically studied. Therefore, we envision that AFM-based SMFS may open a new door toward investigations of the mechanism of infection, cotranslational disassembly, and transmembrane movement of many viral systems.

Acknowledgment. This work was funded by the NSFC Special Fund Program (20844003), the NSFC Key Program (20834003), SRF for ROCS (SEM), and the NSFC International Cooperation and Exchange Program (20640420622). We are grateful to Professor Culver for the TMV1Cys inoculums. Q.W. is grateful for the financial support from the NSF CAREER Program, NSF DMR, DOE-BES, and ARO.

Supporting Information Available: Description of experimental methods and several control measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Harrison, B. D.; Wilson, T. M. A. *Phil. Trans. R. Soc. London, Ser. B* **1999**, *354*, 521, and references therein.
(2) Bawden, F. C.; Pirie, N. W.; Bernal, J. D.; Fankuchen, I. *Nature* **1936**, *138*, 1051.
(3) (a) Namba, K.; Stubbs, G. *Science* **1986**, *231*, 1401. (b) Namba, K.; Pattanayek, R.; Stubbs, G. *J. Mol. Biol.* **1989**, *208*, 307. (c) Stubbs, G. *Phil. Trans. R. Soc. London, Ser. B* **1999**, *354*, 551.
(4) Klug, A. *Phil. Trans. R. Soc. London, Ser. B* **1999**, *354*, 531.
(5) Sachse, C.; Chen, J. Z.; Coureux, P.-D.; Stroupe, M. E.; Fändrich, M.; Grigorieff, N. *J. Mol. Biol.* **2007**, *371*, 812.
(6) Humphrey, W.; Dalke, A.; Schulten, K. *J. Mol. Graphics* **1996**, *14*, 33.
(7) Shaw, J. G. *Phil. Trans. R. Soc. London, Ser. B* **1999**, *354*, 603.
(8) Florin, E.-L.; Moy, V. T.; Gaub, H. E. *Science* **1994**, *264*, 415.
(9) (a) Conti, M.; Falini, G.; Samorì, B. *Angew. Chem., Int. Ed.* **2000**, *39*, 215. (b) Valle, F.; Zuccheri, G.; Bergia, A.; Ayres, L.; Rowan, A. E.; Nolte, R. J. M.; Samorì, B. *Angew. Chem., Int. Ed.* **2008**, *47*, 2431.
(10) Zhang, Y. H.; Liu, C. J.; Shi, W. Q.; Wang, Z. Q.; Dai, L. M.; Zhang, X. *Langmuir* **2007**, *23*, 7911.
(11) Kim, J. S.; Jung, Y. J.; Park, J. W.; Shaller, A. D.; Wan, W.; Li, A. D. Q. *Adv. Mater.* **2009**, *21*, 786.
(12) Hugel, T.; Holland, N. B.; Cattani, A.; Moroder, L.; Seitz, M.; Gaub, H. E. *Science* **2002**, *296*, 1103.
(13) Shi, W. Q.; Giannotti, M. I.; Zhang, X.; Hempenius, M. A.; Schönherr, H.; Vancso, G. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 8400.
(14) Rief, M.; Gautel, M.; Fernandez, J. M.; Gaub, H. E. *Science* **1997**, *276*, 1109.
(15) (a) Li, H. B.; Linke, W. A.; Oberhauser, A. F.; Carrion-Vazquez, M.; Kerkvliet, J. G.; Lu, H.; Marszalek, P. E.; Fernandez, J. M. *Nature* **2002**, *418*, 998. (b) Peng, Q.; Li, H. B. *J. Am. Chem. Soc.* **2009**, *131*, 14050.
(16) Rief, M.; Clausen-Schaumann, H.; Gaub, H. E. *Nat. Struct. Biol.* **1999**, *6*, 346. Cui, S. X.; Yu, J.; Kühner, F.; Schulten, K.; Gaub, H. E. *J. Am. Chem. Soc.* **2007**, *129*, 14710.
(17) Zhang, W. K.; Machon, C.; Orta, A.; Phillips, N.; Roberts, C. J.; Allen, S.; Soultanas, P. *J. Mol. Biol.* **2008**, *377*, 706.
(18) (a) Borgia, A.; Steward, A.; Clarke, J. *Angew. Chem., Int. Ed.* **2008**, *47*, 6900. (b) Williams, P. M.; Fowler, S. B.; Best, R. B.; Toca-Herrera, J. L.; Scott, K. A.; Steward, A.; Clarke, J. *Nature* **2003**, *422*, 446.
(19) For recent reviews, see: (a) Clausen-Schaumann, H.; Seitz, M.; Krautbauer, R.; Gaub, H. E. *Curr. Opin. Chem. Biol.* **2000**, *4*, 524. (b) Hugel, T.; Seitz, M. *Macromol. Rapid Commun.* **2001**, *22*, 989. (c) Janshoff, A.; Neitzert, M.; Oberdörfer, Y.; Fuchs, H. *Angew. Chem., Int. Ed.* **2000**, *39*, 3212. (d) Zhang, W. K.; Zhang, X. *Prog. Polym. Sci.* **2003**, *28*, 1271. (e) Müller, D. J.; Dufrêne, Y. F. *Nat. Nanotechnol.* **2008**, *3*, 261. (f) Hinterdorfer, P.; Dufrêne, Y. F. *Nat. Methods* **2006**, *3*, 347. (g) Müller, D. J.; Helenius, J.; Alsteens, D.; Dufrêne, Y. F. *Nat. Chem. Biol.* **2009**, *5*, 383. (h) Zhuang, X. W.; Rief, M. *Curr. Opin. Struct. Biol.* **2003**, *13*, 88. (i) Allison, D. P.; Hinterdorfer, P.; Han, W. *Curr. Opin. Biotechnol.* **2002**, *13*, 47. (j) Zhang, Q. M.; Lu, Z. Y.; Hu, T.; Yang, W. T.; Marszalek, P. E. *J. Am. Chem. Soc.* **2006**, *128*, 9387.
(20) Royston, E.; Ghosh, A.; Kofinas, P.; Harris, M. T.; Culver, J. N. *Langmuir* **2008**, *24*, 906.
(21) Smith, S. B.; Cui, Y. J.; Bustamante, C. *Science* **1996**, *271*, 795.
(22) Steckert, J. J.; Schuster, T. M. *Nature* **1982**, *299*, 32.

JA1052544