Subangstrom single-molecule measurements of motor proteins using a nanopore

Ian M Derrington1, Jonathan M Craig1, Eric Stava2, Andrew H Laszlo1, Brian C Ross1, Henry Brinkerhoff3, Ian C Nova1, Kenji Doering1, Benjamin I Tickman1, Mostafa Ronaghi2, Jeffrey G Mandell2, Kevin L Gunderson2 & Jens H Gundlach1

Techniques for measuring the motion of single motor proteins, such as FRET and optical tweezers, are limited to a resolution of ~300 pm. We use ion current modulation through the protein nanopore MspA to observe translocation of helicase Hel308 on DNA with up to ~40 pm sensitivity. This approach should be applicable to any protein that translocates on DNA or RNA, including helicases, polymerases, recombinases and DNA repair enzymes.

The ability to directly observe the molecular motion of single molecules in real-time provides insights that are not feasible with bulk assays. To date, the highest-precision single-molecule measurements have been obtained using optical tweezers, which can measure motor protein procession with ~300 pm spatial resolution and time scales of ~1 ms (refs. 1, 2). Here we present single-molecule picometer-resolution nanopore tweezers (SPRNT), a method for monitoring the motion of DNA and conformational changes of processive nucleic-acid-binding proteins as the nucleic acid passes through a nanopore. SPRNT detects nucleic acid motion relative to the enzyme that processes it with a precision of ~40 pm on timescales shorter than a millisecond. We use SPRNT to observe two distinct substrates in the ATP hydrolysis cycle of a helicase.

SPRNT draws upon the concept of nanopore DNA sequencing3. In nanopore sequencing, a nanometer-sized pore is formed between two chambers filled with an ionic solution (Fig. 1a and Supplementary Fig. 1). Upon application of an electrostatic field, an ion current flows through the pore and draws single-stranded DNA (ssDNA) into the pore. The presence of DNA nucleotides in the pore's constriction modulates the ion current. A motor enzyme is used to move the ssDNA through the pore at speeds of 1–100 nt/s, thereby enabling the ion current to be correlated with the DNA sequence4,5. Here we use a mutated Mycobacterium smegmatis porin A (MspA)6, which has a short, narrow constriction capable of resolving individual DNA nucleotides (Fig. 1b)4,7. We find that the exquisite base sensitivity of MspA allows for precise measurement of the position of the DNA inside the pore and thereby enables study of the motion of DNA through processive enzymes. Such measurements can be used to infer conformational changes of enzymes and kinetic stepping parameters. Figure 1c shows raw ion current data. Each current level represents a single-nucleotide (nt) step of the motor enzyme phi29 DNA polymerase (DNAP) along the DNA strand and the current magnitude corresponds to the sequence of the DNA passing through the pore's constriction (Fig. 1d)4,7. Each level can be resolved with submillisecond accuracy and pico-ampere (pA) precision (Supplementary Fig. 2).

For some sequence contexts there is a large change in ion current when the DNA moves by 1 nt. For example, the ion current levels associated with a sequence of DNA containing an abasic site (marked by an "X") has a change in current equal to ~16 pA when the DNA moved by 1 nt (Fig. 1e). If the DNA were to move within MspA by a distance of about one tenth of a nucleotide, a linear interpolation would have the observed current change by about one tenth of the change in current, or ~1.6 pA. Coupling the ion-current to the DNA position allows us to measure the position of DNA in MspA to precision much smaller than 1 nt. The scales in Figure 1c, which show the conversion of current to displacement, use a cubic spline to approximate the ion current between levels measured at 1 nt intervals. Using this distance scale, we relate the uncertainty of ion current levels to the uncertainty of the DNA position in the pore. For the ion current levels depicted in Figure 1c a position uncertainty as small as 0.06 nt can be resolved, corresponding to a distance uncertainty of ~40 pm. We assume an interphosphate distance of 690 pm (refs. 8,9) and 88–95% DNA-elongation.

Next, we changed the elongation of DNA by altering the electrostatic force applied to the DNA. While DNA was moved by phi29 DNAP in single-nucleotide steps, we applied driving potentials of 140 mV and 180 mV. Changing the voltage (and thereby the force on the DNA) alters the elongation of DNA between the motor enzyme and pore constriction and shifts the position of nucleotides within MspA's constriction (Fig. 1f). Figure 1g displays the levels for data taken at the two voltages with cubic spline interpolants overlaid. The location of the splines’ peaks shift between the different voltages. After normalizing the current amplitudes, we find that the spline for levels taken at 180 mV can predict the levels at 140 mV, when the spline is shifted 0.29±0.03 nt (Fig. 1h). Exploring DNA elongation with voltages between 100 mV and 200 mV indicated that the DNA elongation was consistent with experimental force-stretching curves for ssDNA1,9 for forces in the range ~20–40 pN (Supplementary Fig. 3 and Supplementary Discussion 1). These results show that the spline is a reasonable prediction of currents between levels seen at 1 nt intervals.

We evaluated the precision of SPRNT using Hel308 of Thermococcus gammatolerans EJ3 (hereafter Hel308), which is an ATP-dependent Sk2-like superfamily II (SF2) helicase/translocase that unwinds duplex DNA in the 3’ to 5’ direction. Hel308 is conserved in many archaea and eukaryotes, including humans10. With a known crystal structure, Hel308 is a good system for understanding processive SF2 helicases11.

The current patterns we observed were qualitatively similar to those observed with phi29 DNAP (Fig. 2a,b). However, when Hel308 moved...
DNA through the pore, we observed nearly twice the number of levels as compared to when phi29 DNAP moved DNA through the pore even though the same length of DNA passed through the pore (Fig. 2a, b).

By comparing 72 Hel308 DNA translocation events, we produced a consensus set of current levels for Hel308 DNA translocations of DNA “sequence A” through the pore (Fig. 2c, d, Supplementary Discussion 2, and Supplementary Figs. 4 and 5). We used this consensus set to deduce the position of DNA when Hel308 controlled DNA translocation compared with the position of the same DNA sequence moved by phi29 DNAP (Fig. 2a and Online Methods). We found that the odd-numbered Hel308 current levels corresponded to the DNA being held 0.14 ± 0.03 nt higher in the pore than the closest corresponding current level taken with phi29 DNAP. We found the even-numbered Hel308 levels corresponded to the DNA being held 0.41 ± 0.03 nt lower in the pore. The average difference in position between the odd- and even-numbered Hel308 steps is therefore 0.55 ± 0.04 nt (Supplementary Discussion 2 and Supplementary Fig. 6).

Next, we examined the median duration ($\tau_{\text{med}}$) of each level at different ATP concentrations (Fig. 2e and Supplementary Discussion 3). In Figure 2f we compare the median duration of each current level at 10 $\mu$M ATP to those at 1 mM ATP by dividing $\tau_{\text{med}}$ (10 $\mu$M) by $\tau_{\text{med}}$ (1 mM); we found that the durations for even-numbered levels depended on ATP concentration whereas durations for odd-numbered levels were independent of ATP concentration. The ion current magnitude did not change with ATP concentration. (A full ATP concentration titration is described and shown in Supplementary Discussion 4 and Supplementary Figs. 7–9).

Analysis of the crystal structures of Hel308 and the SF2 helicase, Vasa, by Büttner et al.11 revealed large conformational shifts upon ATP binding. Büttner et al.11 proposed an inchworm model in which the two RecA-fold DNA binding domains, through the action of ATP binding and then hydrolysis, take turns moving along a DNA strand. In SPRNT the movement of the DNA in the MspA pore is likely a combination of the movement of DNA inside Hel308 and conformational changes of the Hel308 that reposition Hel308 on the MspA rim (thereby changing the position of DNA inside the pore; Supplementary Fig. 10a). Even so, our observations seem to confirm the model predicted by Büttner et al.11. Using Büttner’s model11, we suggest that motif IV within domain 2 pushes the DNA partially upward within the pore (Supplementary Fig. 10b,c).
ATP hydrolysis and ADP release finishes the hydrolysis cycle advancing the DNA and finishing the single-nucleotide step. Previously, substrate kinetic steps have only been inferred indirectly through fitting of durations in helicase systems12. However, to our knowledge, no other real-time single-molecule method has allowed direct observation of substrates within individual hydrolysis cycles of helicase kinetics.

To maximize SPRNT’s resolution, it is important to choose DNA sequences that produce current levels with large differences (not homopolymeric sequences). The current between full nucleotide steps may differ from the spline interpolation that we used. Finally, during SPRNT, MspA is in contact with the enzyme and applies a 20–50 pN force to the enzyme (Supplementary Discussion 1). These forces and contact with MspA may alter the enzyme’s activity.

In addition to subangstrom resolution, SPRNT simultaneously provides the exact location of the enzyme along the DNA sequence7. This means that SPRNT could be used to answer important questions in many motor enzyme systems, such as how nucleic acid sequence and structure relate to pausing and other motor enzyme activity13. SPRNT can resolve smaller motions of enzyme subdomains than fluorescence resonance energy transfer (FRET) and could be used with DNA and RNA polymerases or translocases, a ribosome or transcription complexes. Other potential applications include analyzing reactive molecules tethered to a polymer (DNA, RNA or hybrids) that is held in the pore.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

J.M.D., J.M.C., E.S., A.H.L., I.C.N., J.G.M., K.L.G. and J.H.G. designed experiments, J.M.C., E.S., A.H.L., B.C.R., H.B. and J.H.G. analyzed the data. J.M.D., J.M.C., A.H.L., J.H.G. wrote the paper. M.R., K.L.G. and J.H.G. led the research teams.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Proteins. The M2-NNN-MspA protein6 was custom ordered from GenScript. Wild-type phi29 DNAP (833,000 U/ml; specific activity 83,000 U/mg) was obtained from Enzymatics or Epicenter. Hel308 was expressed using standard techniques by in-house facilities. Both phi29 DNAP and Hel308 were stored at −20 °C until immediately before use.

DNA constructs. DNA oligonucleotides were synthesized at Stanford University Protein and Nucleic Acid Facility and purified at their facility using column purification methods. The oligo sequences are shown in Supplementary Table 1. For both phi29 DNAP and Hel308 experiments, the nanopore read the same sequences for DNA threaded 5′ → 3′ first. For phi29 DNAP experiments, sequences were previously used4,14. In particular, DNA templates, primers and blocking oligomers were mixed at relative molar eight base 3′ overhang on the template to a complement primer such that the template strand had an 18 base 3′ overhang on the template strand and may begin to unwind the dsDNA in the 5′ direction. The 5′ end of the template DNA strand is drawn into the pore by the voltage, causing the complementary strand to dissociate.16. Hel308 bound to the DNA prevents complete translocation of the template strand through MspA17. Hel308 then functions as a translocase, drawing the ssDNA out of the nanopore in the 3′ direction back into the cis well. Supplementary Figure 3 illustrates the DNA translocase activity of DNA of through MspA by Hel308. We recorded >2,000 current traces in various conditions, demonstrating enzymatic movement along DNA (Supplementary Table 2). With 180 mV applied, the currents were higher with Hel308 than with phi29 DNAP because the buffer contained higher [KCl].

Operating buffers. For phi29 DNAP experiments we used buffers of 300 mM KCl or asymmetric 150 mM cis KCl and 50 mM trans KCl both with 10 mM HEPES at pH 8.0, 1 mM EDTA, 1 mM DTT and 10 mM MgCl2. For Hel308 we used buffer at 400 mM KCl with 10 mM HEPES at pH 8.0, 1 mM EDTA, 1 mM DTT and 10 mM MgCl2. Buffer [KCl] was higher than for phi29 DNAP experiments because the helicase operated better in higher salinity conditions.

Nanopore experiments. The experiments containing single M2-NNN MspA nanorods were established using thoroughly established techniques6–18. In short, we formed a lipid bilayer with 1,2-diphytanoyl-sn-glycerol-3-phosphocholine (Avanti Polar Lipids) across a horizontal ~20 µm diameter aperture separating two ~60 µl chambers containing our operating buffers. (Supplementary Fig. 1). An Axopatch 200B or Axopatch 1B integrating patch clamp amplifier (Axon Instruments) applied a 180 mV voltage (unless otherwise noted) across the bilayer (trans side positive) and measured the ionic current through the pore. M2-NNN MspA was added to the grounded cis compartment to a final concentration of ~2.5 ng/ml. Once a single pore inserted, as seen by a characteristic increase in the conductance, the buffer was replaced with MspA-free buffer to prevent additional pore formation. The DNA was added to the cis compartment to a final concentration of 10 nM. In a standard phi29 DNAP experiment dCTP, dATP, dTTP and dGTP was added at the final concentrations of 100 µM and phi29 DNAP was added to a final concentration 20 nM. In standard Hel308 experiments, our buffers of 400 mM KCl were premade with varying concentrations of ATP (10 µM, 20 µM, 50 µM, 250 µM, 500 µM, 1 mM, 3 mM). 1 ml of the chosen premixed solution was perfused into the cis chamber, ensuring the uniform concentration of ATP. In the Hel308 experiments, DNA was added to a final concentration of 10 nM and Hel308 to a final concentration of 100 nM. Unless otherwise mentioned, experiments were done at room temperature (23 ± 1 °C).

Measurement of DNA position for Hel308 experiments. After scaling Hel308 levels and positioning them relative to the levels previously measured with phi29 DNAP, we found that all Hel308 levels lie along a spline interpolant between the phi29 DNAP current levels (Fig. 2c). Odd-numbered Hel308 levels are close to previously observed phi29 DNAP current levels. Even-numbered Hel308 levels lie along the interpolant somewhere in-between previously measured levels. As in Figure 2d, we found the position of both the even- and odd-numbered Hel308 levels relative to the levels taken with phi29 DNAP.

Data acquisition and analysis. Data were acquired at 50 kHz with acquisition software written in LabView (National Instruments). Current traces were analyzed using custom programs written in Matlab (The MathWorks), Java and C. Collected data were box-filtered with at 10 point window and downsampled to 5.0 kHz. DNA interactions and enzyme motor events were detected using previously described algorithms4,6,7,14,19. Ion current levels were selected automatically using a level-finding algorithm7, using elements of the level finder more thoroughly described in Schreiber et al.20. Event counts and statistics are summarized in Supplementary Table 2. Additional methods can be found in the Supplementary Discussions 1–4.

Protein accession numbers. Hel308 of Thermococcus gammatolerans EJ3 (WP_015858487.1). Our MspA pore is identical to wild-type MspA (CAB56052.1) except for the following modifications: D90N/D91N/D93N/D118R/E139K/D134R. Phi29 DNAP from bacteriophage phi29 (P03680.1).

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