Helicase SPRNTing through the nanopore

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Enzymes that move directionally on single-stranded nucleic acids are at the core of emerging nanopore sequencing technology. Of a particular use are DNA helicases, molecular motors that bind single-stranded DNA (ssDNA) independently of its sequence and use ATP to fuel their directional motion along the DNA (1). In nanopore-based sequencing, a pore formed by a protein channel embedded into a lipid membrane forms an electrical connection between two salt solutions; electrostatic potential applied across the nanopore drives individual ssDNA molecules through the nanopore aperture; the helicase bound to the DNA controls the ssDNA movement by either pulling the DNA out of the nanopore or by feeding it into the nanopore one nucleotide at a time. The ion current through the nanopore reveals the DNA sequence, while changes in this current reflect the ssDNA movement within the helicase (2, 3). Choosing the right enzyme and utilizing its full potential in the nanopore-based sequencing require a detailed understanding of the helicase’s mechanochemical cycle. A report in PNAS (4) delves into a complex mechanism of the ssDNA translocation by HEL308 DNA helicase. The results of the reported investigation offer intriguing insights into the helicase translocation mechanism, as well as information important for improving the nanopore sequencing methodology.

Since the discovery that charged molecules, such as ssDNA, could be electrophoresed through an ion channel in a lipid bilayer (5), nanopore technology has developed into a robust method of nucleic acid sequencing (2, 3). Nanopore sequencing utilizes a pore placed in a phospholipid bilayer separating two electrolyte solutions. A voltage applied across the membrane generates an ion current, which drives single-stranded oligonucleotides, ssDNA or ssRNA, through the pore (6). A helicase or polymerase that is bound to the oligonucleotide feeds the nucleic acid strand into or pulls it through the channel in single-nucleotide steps (Fig. 1). Helicases offer an advantage over polymerases as they bind single-stranded nucleic acid molecules at and initiate movement from random positions along the lattice, while polymerases require a partial duplex where the new nucleotides are added to the 3’ end of the primer. Because of the polar nature of single-stranded nucleic acids and because of the directional nature of the helicase movement on their respective substrates (some helicases move with 3’-to-5’ directionality, while others move 5’ to 3’), whether the ssDNA will be pulled through the nanopore with the current or against it depends on which end of the molecule enters the nanopore first. As the oligonucleotide is progressing through the pore, the ion current fluctuates in a manner dependent upon the nucleotides that are within the channel. The fluctuations in current are then analyzed to determine the sequence of the polynucleotide (6).

Several challenges come with the optimization of nanopore sequencing. The first challenge presents itself in detection of the sequence. Ion current fluctuations used to determine the sequence depend on how...
on stretches of several nucleotides within the region of base sensitivity in a particular pore. In pores with larger regions of base sensitivity, ~12 nt in the case of α-hemolysin, single-nucleotide resolution cannot be achieved (6, 7). A modified *Mycobacterium smegmatis* porin A (MspA), which has a 4-nt region of base sensitivity at the nanopore aperture, can provide the single-nucleotide resolution required for sequence detection (6, 8). The same applies to the CsgG bacterial amyloid secretion pore commercialized by Oxford Nanopore Technology in their MiniON device (9). Another important challenge is controlling the motion of the oligonucleotide through the channel. Simply applying voltage across the membrane will drive single-stranded nucleic acids through the channel too rapidly. With current as the sole driving force, each nucleotide spends less than 10 µs in the pore, while more than 100 µs is needed to achieve an adequate signal-to-noise ratio (10). Using molecular motors, such as helicases, allows for an increased level of control (10, 11). While these motors decrease speed, they come with other limitations, such as allowing both forward and reverse steps. Optimizing the molecular motor is essential to optimizing nanopore sequencing techniques.

A number of studies in recent years utilized near-base pair resolution, single-molecule techniques to discern the mechanisms of the namesake activity of various helicases, that is, the helicase-mediated separation of the DNA or RNA double helix (12–14). A complicated kinetics of forward and reverse steps and transitions between alternative conformational states were observed. It has been assumed, however, that this complicated stepping kinetics is due to the differences in stability between different sequence contexts ahead of the helicase and different degrees of passivity in the duplex unwinding by different helicases. In the absence of secondary structure, the ssDNA translocation by all helicases was presumed to be sequence independent. This expectation has been especially firm for the superfamily 2 (SF2) helicases, whose motor cores interact mostly with the phosphodiester backbone of the translocating nucleic acid.

In an elegant study in *PNAS*, Craig et al. (4) applied single-molecule picometer resolution nanopore tweezers (SPRNT), a high-resolution, nanopore-based single-molecule approach to study the ssDNA translocation of HEL308 DNA helicase from *Thermococcus gammadotolerans* (Fig. 1). This work builds on the previous publication by the authors (15) that showed the ability of SPRNT to resolve subnucleotide steps of the DNA-translocating motors. Here, the high resolution afforded by SPRNT (the ion current through the MspA pore is converted into a measurement of DNA position with ~40-PM spatial resolution and on a millisecond timescale) allowed the authors to clearly observe and quantitatively evaluate the individual forward and backward steps of HEL308 over a broad range of experimental conditions. SPRNT recordings of thousands of HEL308 forward and backward translocation steps were analyzed to build a comprehensive kinetic model. The first exciting revelation of the work was the presence of the half-nucleotide substeps in the mechanochemical cycle of HEL308, where one half-step displayed a Michaelis–Menten dependence on the ATP concentration, while the second half-step was nucleotide concentration independent. The motor cores of the SF2 DNA helicases, such as HEL308, are composed of two RecA-like domains. Each domain interacts with ~5 consecutive nucleotides. An ATP molecule binds between the two RecA-like domains and drives or rectifies the mechanochemical cycle: ATP binding, hydrolysis, and release of ADP and inorganic phosphate modulates the interactions between the helicase and the ssDNA, as well as the relative distance between the two RecA-like domains, resulting in "inchng forward" by the helicase by 1 nt during each cycle of ATP binding and hydrolysis (16). In the case of HEL308, these forward steps are taken in the 3′-to-5′ direction with occasional backward missteps. The authors propose two plausible models that explain the substeps. In the first model, the [ATP]-independent step occurs between ATP binding and ADP release, and involves the ATP hydrolysis and the sequence of conformational changes. The ATP-bound state favors the conformation that results in the half-nucleotide motion forward, while the ADP-bound conformation favors a backward motion by a half-nucleotide. Notably, the presence of substeps is also integral to the mechanism of another ATP-driven motor, the F1 ATPase (17). Per each ATP binding and hydrolysis cycle, the central γ-subunit of the F1 ATPase rotates within the ATPase ring in discrete 120° steps where each step consists of two substeps, the 90° step driven by ATP binding and the 30° step driven by the release of ADP and inorganic phosphate. The mechanochemical cycle of F1 ATPase is fine-tuned to enable the most efficient energy conversion. It is interesting therefore to see similar substeps in an enzyme that is much less efficient with respect to the free energy extracted from ATP binding and hydrolysis.

The second intriguing observation in this current SPRNT study is the sequence dependence of the translocation kinetics. While every ssDNA–HEL308 complex entering the nanopore may have the helicase bound to a random position along the ssDNA molecule, SPRNT allows the simultaneous measurement of the dwell times in the substrates and the identification of the bases at the MspA aperture, and thus defines the DNA position-dependent kinetics. However, it is unclear how many nucleotides lie between the MspA region of base sensitivity and the structural features of the helicase that may control the translocation rate in a sequence-dependent manner. Similar to that of other SF2 helicases, the motor core of HEL308 makes contacts with the ssDNA phosphodiester backbone and therefore is unlikely to display sequence dependence in DNA translocation. Some SF2 helicases, such as XPD, have secondary DNA binding sites that control the helicase’s rate (18), although it is unclear whether this control is exerted at the level of translocation or duplex unwinding and whether base-specific contacts are involved. The auxiliary so-called ratchet domain of HEL308 makes several contacts with the ssDNA that involve bases and therefore may offer determinants of the potential sequence specificity (19). The nature of the structural and mechanistic determinants of the sequence specificity in HEL308 and whether it extends to other helicases remains a mystery and will require future studies to solve, with SPRNT providing a robust and convenient platform for such investigations.

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