New and Notable

Sequencing and the Single Channel

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Single-channel electrophysiology provides for high-resolution measurements of the dynamics of single macromolecules, the channel proteins (Neher and Sakmann, 1976). Among the measurements that have proven fruitful have been ability to monitor, in almost real time, the interactions between the channel and a blocker, or modifier, of ion permeation (Neher and Steinbach, 1978; Prod'hom et al., 1987; Hemsley and Busath, 1991). The emphasis usually has been on the channel, but different blockers/modifiers may have different characteristic "fingerprints" (Hemsley and Busath, 1991), which provides for possibilities in terms of using the characteristics of the blocking events to determine the chemical identity of the blocking species.

In this issue of *Biophysical Journal*, Akeson et al. explore the feasibility of using single ion channels, in this case the *Staphylococcus aureus* α-hemolysin channels, to determine the chemical identity of polynucleotides, with the aim of establishing a method for DNA or RNA sequencing. Earlier work (Kasianowicz et al., 1996) showed that single-stranded DNA or RNA molecules can cause brief, incomplete block of the current through α-hemolysin channels when the polarity of the applied potential is such as to drive the polynucleotides into the pore. Kinetic analysis provided evidence for three different kinds of incomplete blocking events. When examining the relation between polynucleotide length and block duration, the average durations of two of the three types of blocking events varied as linear functions of the polynucleotide length, suggesting they could be related to polynucleotide passage through α-hemolysin channels. If so, the average block duration should decrease as the magnitude of the applied potential increased, which was observed. To validate the suggestion, Kasianowicz et al. (1996) used quantitative polymerase chain reaction to demonstrate that single-stranded, but not double-stranded, DNA could pass through α-hemolysin-doped bilayers. The flux of single-stranded DNA through the bilayer, as evaluated by the polymerase chain reaction, could be related quantitatively to the frequency of long-lived blocking events. Moreover, polynucleotides that were constructed such that they would have both single- and double-stranded stretches caused long-lived current blocks, which could be "cleared" by reversing the polarity of the applied potential. Finally, the frequency of blocking events caused by RNA varied predictably when ribonuclease was added to the polynucleotide-containing solution.

Do individual polynucleotides have distinct signatures that reflect their chemical composition, and are these signatures sufficiently different that one might be able to use single-channel methods for sequencing purposes? As shown by Akeson et al., the answers are "yes" and "most likely." When examining blocking events caused by homopolynucleotides, one can distinguish the characteristic current reduction (~85%) caused by the passage of polyadenylic acid (poly A) from the current reduction (90–95%) caused by the passage of polycytidylic acid (poly C). This difference in current levels allows Akeson et al. to detect the passage of tandem polynucleotides, such as a construct with 30 A and 70 C, which is very encouraging for future developments.

But then matters get complicated. Polyuridylic acid (poly U), for example, causes a current reduction similar to that caused by poly A. The kinetics of polynucleotide transfer through the α-hemolysin channel are different, however, as the passage of poly U (~1.4 μs/nucleotide at 120 mV) is about 10-fold faster than that of poly A (~20 μs/nucleotide) for polynucleotides of comparable length. Because the distribution of blocking durations (polynucleotide passage times) is nonexponential and appears similar to a normal distribution (Kasianowicz et al., 1996), one might be able to distinguish between poly A and poly U based simply on their characteristically different passage times, assuming polynucleotides of equal length.

Complications also arise because the secondary structure of the polynucleotides influences both the magnitude and time distribution of the current reductions. The pyrimidine cytosine, for example, is less bulky than the purine adenosine. Even accepting the lack of a well-defined relation between lumen geometry and conductance, e.g., (Finkelstein, 1985), one therefore would expect a larger current reduction when poly A traverses the channel than when poly C does so, which is contrary to what is observed. Under "normal" conditions, however, poly C occurs primarily as single-stranded helices, with a diameter (~1.3 nm) that is close to the luminal diameter of the α-hemolysin pore (~1.4 nm; Song et al., 1996). One therefore might surmise that the more organized single-stranded structure could cause a lower conductance and a longer passage time per nucleotide, ~6 μs, than that of the more disorganized poly U. That appears to be the case, as polydeoxycytidylic acid (poly dC), which has less secondary structure, causes an only 85–90% current reduction and traverses the channel at a rate of ~1 μs

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per nucleotide. The concern about secondary structure becomes even more acute in the case of poly A, which again forms single-stranded helix, with a diameter of ~2 nm. In this case, the poly A structure would need to unfold for the polynucleotide to traverse the channel, which might account for the much longer passage times observed for poly A. In fact, poly dA traverses the channel at a rate of ~3 μs per nucleotide, about 5-fold faster than poly A, consistent with the notion that the rate of polynucleotide passage (and maybe even the magnitude of the current reductions) may be biased by the secondary structure of the polynucleotide.

However, if the polynucleotides have significant secondary structure, how does the channel catalyze the unwinding of the secondary structure at a rate sufficient to account for the observed kinetics of the blocking events? That remains an open question. The rate constant for the polynucleotide-induced block of the α-hemolysin channels has yet to be determined; however, Kasianowicz et al. (1996) reported that the blocking rate for equimolar polynucleotide concentrations was faster for shorter (~200 nucleotides) than for longer (~1000 nucleotides) polynucleotides, suggesting that polynucleotide unfolding and orientation is important. Given the results in Table 1 of Kasianowicz et al. (1996) the bimolecular rate constant for passage of polydeoxynucleotides with ~150 nucleotides is $\sim 10^4\ M^{-1}{\cdot}s^{-1}$, which should be comfortably below the diffusion-controlled limit and thus allow for significant "processing" of the polynucleotide as it associates with the pore entrance. The time course of any such processing should depend of the orientation of the partially structured polynucleotide in relation to the pore entrance, which might provide insights into why the distribution of passage times through the pore is much broader than predicted from "simple" polymer theory (Lubensky and Nelson, 1999).

The results of Akeson et al. are very promising, but the question remains of how close we are to polynucleotide sequencing by single-channel methods. Two quite different limitations need to be overcome before one can construct a prototype. First, how do we overcome the limitations imposed by the secondary structure dependence? It may be possible to do the experiments at temperatures sufficiently high to disrupt the secondary structure, which will become particularly important for "real" polynucleotides that may form double helices, which would block polynucleotide transfer through the pores (Kasianowicz et al., 1996). Gawrisch et al. (1992), in fact, could form bilayers from dipalmitoylphosphatidylcholine at ~50°C, which might be sufficient to eliminate the secondary structure problem. Second, how do we deconvolute the current signal to give information about the underlying nucleotide sequence? Given appropriate reference data, similar to those provided by Akeson et al., it should be possible to use computational methods similar to those that have been developed for extracting idealized single-channel current traces from noisy current records (Venkataramanan et al., 1998). Even then, however, one probably would need to slow down the polynucleotide transfer through the pore in order to get a satisfactory signal, which creates demands that seem to be orthogonal to the need for disrupting the secondary structure, unless one could use the slowing-down caused by unwinding the secondary structure productively. A saving grace may be that the atomic resolution structure of the α-hemolysin channel is known, which means that one may be able to introduce bulky residues that would slow down the passage through the pore, and maybe even catalyze the unraveling of any secondary structures.

Eventually the bilayer technology is likely to become limiting, as it will be difficult to design a bilayer-based apparatus that would be sufficiently stable for routine sequencing purposes. Polymerizable lipids may offer a solution, but technical problems remain to resolved (Rhodes et al., 1994). Alternatively, one might be able to use nano-scale pores etched in irradiated micro-fabricated materials, as this allows for pore diameters close to what would be needed (Bean et al., 1970) and has proven useful for the sizing of viruses (DeBlois and Wesley, 1977).

Finally, how are other single-molecule methods for polynucleotide sequencing progressing? Currently, no fully implemented method exists, but the future is almost here. Eigen and Rigler (1994) proposed a method for DNA sequencing based on single-molecule detection using fluorescence correlation microscopy using exonuclease digestion of DNA that was synthesized using fluorescence-labeled nucleotides, and Kinjo et al. (1998) showed how one can monitor the restriction enzyme cleavage of such fluorescent DNA fragments. Recently, at the 13th International Biophysics Congress in New Delhi, India, M. Gösch (Gösch et al., 1999) reported on the controlled digestion of labeled DNA, which was fixed by laser tweezers in a microstructure with fast hydrodynamic flow, and detection of the released nucleotides by their fluorescent signature. Work needs to be done, but a practical implementation is likely to appear in the near term. These different methods are likely to be complementary, however, and the electrophysiological approach proposed by Kasianowicz et al. (1996) and Akeson et al. allows, in principle, sequencing of unmodified polynucleotide fragments.

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