New and Notable

Lighting Up Single Ion Channels

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The holy grail of ion channel studies is to produce an atomic scale movie of an ion channel at work, simultaneously observing conformational and electrical changes as ions flow through the protein. Significant milestones toward this goal include the x-ray crystallography work of Mackinnon et al., which gave us atomic-resolution snapshots of potassium (Zhou et al., 2001) and chloride channels (Dutzler et al., 2002), and patch clamping, which revolutionized the field by giving us the ability to measure ionic currents through single ion channels. Via patch-clamping, kinetics and transitions from different states that affected ionic flow could be detected, thereby revealing a plethora of opened and closed states. Because ion channels stochastically open and close, many of these states could only be revealed by single molecule measurements: measuring ensembles of channels yields only average properties of the open and closed states. Another advance was measuring not ionic currents but gating currents at the ensemble (Armstrong and Bezanilla, 1973) and near single molecule level (Conti and Stuhmer, 1989). Gating currents arise from the movement of charged residues in the protein that move across the membrane electric field. In addition, site-directed mutagenesis, such as cysteine or alanine scanning mutagenesis, gave researchers the ability to introduce point mutations at will and combined with patch clamping, enabled the dissection of how a mutation at a selected position affected these electrical states.

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Despite the power of these techniques, the holy grail remains far off. X-ray studies give a predominantly static picture. Patch clamping of single channels gives information only about those residues whose mutants have an effect on ionic current, and at that, is an indirect look. Analyzing gating current primarily provides insight about residues that are charged and move across the membrane electric field. Hence the desire for more techniques.

Fluorescence is a technique that can overcome some of these limitations while having its own set of problems. The general idea is to insert a cysteine at the desired position within the channel protein and label it with a fluorescent dye. The dye's emission, which can report on the local environment during channel operation, is measured while simultaneously measuring the ionic (or gating) current to identify the functional state of the channel (Mannuzzu et al., 1996; Cha and Bezanilla, 1997). Interpreting the fluorescence changes in terms of protein conformational changes, however, can be complicated due to their indirect relationship. A technically more complex but more informative version of fluorescence is resonance energy transfer (FRET). FRET involves using two dyes: a donor fluorophore transfers energy in a strongly distant—and orientation-dependent—fashion to an acceptor fluorophore. Hence the distance, or possibly orientation, of the two dyes, bound to unique cysteines in the protein, can be measured, yielding information about protein conformational changes. At the ensemble level, FRET has been used to detect conformational changes—a twisting motion—associated with gating in the *Shaker* potassium channel (Glauner et al., 1999; Cha et al., 1999). Extending FRET to the single molecule level (Weiss, 2000) has the distinct advantage that motions which are not correlated between different ion channels in an ensemble, as is the norm in ion channel work, can be revealed.

This is exactly what Borisenko et al. have reported in this month's *Biophysical Journal* (Borisenko et al., 2003). In a proof-of-principle experiment, Borisenko et al. have detected single-pair FRET (spFRET) within a single gramicidin A ion channel embedded in a lipid bilayer, while simultaneously recording ionic currents through the channel. Gramidicin is a dimer, composed of two polypeptides that can associate to form a conducting channel through the membrane, allowing current to flow under an applied transmembrane voltage. Borisenko et al. labeled one peptide with a donor Cy3 dye, and the other with an acceptor Cy5 dye. Two different gramicidin peptides were used, which form channels of low and high conductance as homodimers and intermediate conductance when formed as a heterodimer. Hence, the formation of heterodimers could be identified functionally via electrophysiological measurement, and structurally, via a FRET signal.

Obtaining a FRET signal from just one heterodimer in a bilayer required carefully controlled conditions. To increase the probability that just one heterodimer formed in the lipid bilayer at a time, dilute conditions were needed. Yet because of the limited association constant between the polypeptides, this meant gramacidin monomers, which are nonconducting but still contain single dyes, outnumbered dimers by 100:1. Furthermore, both hetero- and homodimers formed. Hence the FRET signal—most readily identified by an increase in acceptor emission due to energy transfer from an excited donor—had to be measured in a large fluorescent background. Optically, the authors resolved the signal from background in two ways. First the density of channels was made low enough that individual channels could be identified by imaging the lipid bilayer. Second, they preferentially excited the Cy3 such that direct excitation of the Cy5 acceptor was negligible, and also took advantage of the fact that Cy5

emission (far red) is spectrally distinct from Cy3 emission (orange). Hence, any far-red (Cy5) emission indicated FRET was occurring. Consequently by recording electrical currents, noting when a characteristic conductance indicated a heterodimer formed (and unformed), and looking for a spot emitting in the far-red which formed (and unformed) at the same instant, the simultaneous optical and electrical detection of ion channel formation was achieved.

Not surprisingly, the fluorescence and electrical measurements did not always correlate. Sometimes electrical measurements indicated formation of a heterodimer but no FRET signal was seen. This could be due to peptides labeled with optically inactive dyes (due to photobleaching or isomerization), or to channels formed outside the reliable imaging area. At other times FRET was seen but no current was evident, perhaps indicating nonfunctional dimeric structures. While this complexity adds richness to the data, if the correlation becomes too weak one wonders about the utility of the assay.

There are certainly many improvements—both optically and electrically—which can be envisioned for ion channel measurements in particular, and for single-molecule fluorescence in general. Indeed, a number of these are noted by the authors themselves. For example, many channels aggregated near the lipid membrane edge and the limited FRET signal to background prevented a detailed statistical analysis of correlations between spFRET changes and electrical changes upon channel formation. More generally, the photostability of dyes is typically in the seconds time scale, greatly limiting the amount of data from a single channel. In addition, because of limited fluorescence emission rates, the time resolution of single molecule fluorescence studies is milliseconds or longer (5 ms in the Borisenko et al. work), much slower than electrical measurements. An improved understanding of dye photophysics can potentially improve both the speed and photostability of fluorescence, and photostability lifetimes have recently been extended to several minutes (unpublished data). Fluorophores are also fairly large (typically about a nanometer across) and can only be specifically labeled at surface-exposed cysteine residues. Finally, FRET is a residue-by-residue type measurement, far from the ideal of an atomic resolution movie of the entire protein moving. Nevertheless, the work of Borisenko et al. is significant because spFRET and electrical measurements have the potential to add significantly to our understanding of ion channel conformational changes—not the full-blown movie, perhaps, but at least some enticing previews.

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