Protein toxins from venomous organisms have provided remarkable insights into the structure and mechanism of ion channel proteins (Kalai et al., 2015). One classic example is the work of Rod MacKinnon, Chris Miller, and their colleagues on the mechanism by which high-conductance calcium-activated potassium channels (or BK channels for Big K+ conductance) are inhibited by charybdotoxin (Anderson et al., 1988; MacKinnon and Miller, 1988)—a small protein toxin found in the venom of the Israeli deathstalker scorpion, Leirus quinquestratius (Miller et al., 1985). They imagined at the time that the high single-channel conductance of BK might reflect a whirlpool-like structure, and lore has it that a young Gary Yellen made the winning suggestion to name the toxin after Charybdis, the daughter of Poseidon who was turned into a whirlpool-production to name the toxin after Charybdis, the daughter of Poseidon who was turned into a whirlpool-producing sea monster by Zeus. In this issue of The Journal of General Physiology, Daniel Turman and Randy Stockbridge revisit these timeless experiments during their investigation of the mechanism by which monobodies inhibit Fluc fluoride channels. Like MacKinnon, Miller, and colleagues, they find that channel inhibition occurs via a pore block mechanism.

In their pioneering experiments, MacKinnon and Miller used powerful single-channel recordings to show that charybdotoxin binds to BK with bimolecular kinetics expected for a 1:1 complex. Moreover, even though the toxin binds to the extracellular side of the BK channel, they discovered that the toxin unbound more rapidly when they increased the concentration of internal potassium ions or depolarized the voltage across the membrane. Remarkably, this voltage-dependent toxin knock-off vanished when potassium ions were replaced with impermeant ions such as sodium, suggesting that the influence of membrane voltage was to increase potassium occupancy of an ion-binding site near the external side of the pore where the toxin binds. They reasoned that such observations could be understood if charybdotoxin inhibited the channel by physically blocking the pore, as illustrated in the cartoon depicting this bold conclusion from their 1988 JGP paper (MacKinnon and Miller, 1988), reproduced here in Fig. 1 A. This now famous cartoon also speculates that the toxin positions a basic residue at the external end of the permeation pathway, enabling the toxin to sense ion occupancy of the pore. With such pore-blocking scorpion toxins in hand, MacKinnon and Miller localized the pore-forming region within the Shaker potassium channel (MacKinnon and Miller, 1989). MacKinnon demonstrated that this potassium channel is a tetramer (MacKinnon, 1991), and together with Rick Aldrich and Alice Lee, they revealed that one N-terminal inactivation particle was sufficient to inactivate the Shaker channel (MacKinnon et al., 1993). The structural depiction of the toxin inserting a basic residue into the pore gained further traction when Chul-Seung Park and Miller succeeded in producing charybdotoxin recombinantly and showing that mutation of K27 on the toxin greatly reduced the ability of internal potassium or membrane voltage to destabilize the toxin-channel complex (Park and Miller, 1992). Recently, Anirban Banerjee and MacKinnon obtained an even clearer picture when they solved the x-ray structure of charybdotoxin bound to a voltage-activated potassium channel known as the Kv1.2/2.1 paddle chimera and showed that K27 is indeed positioned near the ion selectivity filter within the outer pore of the channel (Fig. 1 B; Banerjee et al., 2013).

In the work described in this issue, Turman and Stockbridge (2017) hark back to the classical studies on charybdotoxin block of potassium channels by demonstrating that a synthetic monobody inhibits the Fluc family of fluoride channels through a remarkably similar mechanism. Flucs are an interesting and recently discovered family of fluoride channels that bacteria, archaea, single-celled eukaryotes, and plants use to rid themselves of toxic fluoride ions found ubiquitously in nature (Baker et al., 2012; Stockbridge et al., 2013; Ji et al., 2014). Fluc subunits come in two varieties, those encoded by a single subunit containing four predicted
transmembrane (TM) helices and those containing two tandem repeats of that basic subunit design, reminiscent of the gene duplication seen between voltage-activated potassium channels and the pseudotetrameric voltage-activated calcium and sodium channel families. Stockbridge and Miller originally suggested that Fluc channels are dimers that assemble in an antiparallel fashion, in part because the naturally occurring tandem-dimer Flucs have an extra TM between the two repeats (Stockbridge et al., 2013).

To develop tools for studying this new class of ion channels, Stockbridge and Miller teamed up with Shohei Koide to generate “monobody” binders. Monobodies are small synthetic proteins based on the 10th fibronectin type III (FN3) domain of human fibronectin (Koide et al., 2012). Originally designed to be used as crystallization chaperones, FN3-based monobodies are similar in structure to immunoglobulin domains of antibodies and can be diversified in the laboratory to form surfaces for molecular recognition (Koide et al., 2007; Koide, 2009). Because of their pocket-sized construction, monobodies often target functional sites on proteins and act as modulators or inhibitors of protein activity (Tanaka et al., 2015). Thus, while Stockbridge and Miller were girding up for crystallization experiments, they exploited the Fluc-targeted monobodies as toxin-like tools to functionally probe the channels’ structure and mechanism.

Fluc channels are constitutively open when reconstituted into planar lipid bilayers, and single-channel experiments revealed that several of the monobodies completely inhibit ion conduction and follow simple bimolecular kinetics (Stockbridge et al., 2014), reminiscent of charybdotoxin block of the BK channel. One of the first elegant uses of these monobodies was to firmly establish the antiparallel assembly of Fluc dimers by showing that they could bind and inhibit single Fluc channels from either side of the membrane (Stockbridge et al., 2014). The eventual high-resolution structures of the Bpe Fluc channel (from Bordetella pertussis), in complex with three distinct monobodies, upheld the antiparallel dimer construction (Stockbridge et al., 2015). Moreover, these new structures provided a surprising new paradigm for ion-channel construction, revealing that the two small (∼15 kD) subunits arrange themselves in antiparallel fashion to form two F−-selective pores in parallel (Fig. 2; Stockbridge et al., 2015; Last et al., 2016). Ironically, the closest architectural relative may be the CLC family of double-barreled chloride channels and transporters (Ludewig et al., 1996; Middleton et al., 1996; Jentsch, 2015; Miller, 2015), in which parallel CI−-selective pathways are formed within individual subunits of a homodimer (rather than along the dimer interface as observed with the Fluc channels). Thus, in short order, the toxin-like monobodies targeting Fluc channels revealed themselves to be powerful tools for studying this intriguing family of new ion channels.

One of the enigmatic features of these remarkable monobodies has been the mechanism by which they
inhibit ion conduction in Fluc channels. Do they work through an allosteric mechanism to stabilize a nonconductive conformation, or might they use a charybdotoxin-like mechanism to physically block ion permeation? Each monobody effectively seals off (i.e., blocks) the permeation pathways in the x-ray structures of the Bpe Fluc (Stockbridge et al., 2015), but it is possible that monobody binding also alters the structure of the channel. In the present study, Turman and Stockbridge (2017) explore the mechanism of monobody inhibition of the Bpe Fluc channel by following up on two interesting and unexplained observations with one of the monobodies, named L3 (Turman et al., 2015). First, membrane depolarization promotes dissociation of the L3 monobody, reminiscent of what was seen earlier with charybdotoxin, but in this case through an unknown mechanism (Turman et al., 2015). Second, L3 monobody binding from one side of the membrane can feel the presence of the monobody bound to the other side, as indicated by a 10-fold weakening of the apparent binding affinity (Turman et al., 2015). This negative cooperativity could be explained by an allosteric mechanism wherein the monobody alters the structure of the Fluc or by an electrostatic repulsion between oppositely bound pore-blocking monobodies, each having a net negative charge of $-4$. Turman and Stockbridge (2017) begin by modeling the L3–Bpe interface based on the L2–Bpe structure because the two monobodies differ at only a few positions. They find that both L2 and L3 have two conserved acidic residues (D28 and E29) that would be candidates for interacting with residues in Bpe. D28 would be expected to form a salt bridge with R68 on Bpe, and mutation of this residue weakens binding of the monobody to such an extent that binding can no longer be detected. In contrast, mutation of E29 has only modest effects on monobody affinity, suggesting that it does not take part in critical interactions with the Fluc channel. However the L3-Bpe model reveals an interesting aspect to the story because it shows that a unique acidic residue in this monobody (E79) projects into one of the two ion permeation pathways (Fig. 2), conjuring up images of K27 in charybdotoxin (Fig. 1 B).

Realizing what they have in their hands, Turman and Stockbridge (2017) proceed to carefully study the E79Q mutant and find that it weakens the affinity of the monobody, but to an extent that does not preclude study of the monobody–Bpe interaction. They carefully look at the kinetics of the E79Q mutant binding to Bpe Fluc and see that it still exhibits bimolecular kinetics and that the weakened affinity of the mutant is caused by a decrease in the lifetime of the L3–Bpe complex. Knowing that the mutant hasn’t fundamentally altered

![Figure 2. Model of the Bpe Fluc channel with L3 monobodies bound. Ribbon representation of a model of the Bpe Fluc channel in complex with L3 monobody, constructed using the x-ray structure of Bpe Fluc in complex with L2 monobody (PDB accession no. 5FXB). Two ion permeation pathways in the antiparallel dimer are positioned lateral to the critical Phe residues (F82 and F85; yellow stick). Each L3 monobody (blue) inserts the FG loop into the pore, with E79 shown in stick representation (carbon colored yellow and oxygen red).](image)
the nature of the monobody–Bpe interaction, they delve deeper into what may have changed in the mutant. They initially find that, although the ability of voltage to promote dissociation of L3 is retained in the relatively innocuous E29Q mutant, it completely vanishes in the E79Q mutant. This is a key result because it predicts the specific conclusion that an acidic residue at position 79 is required for voltage to dislodge the toxin. Second, they reexamine the negative cooperativity that motivated the entire study and obtain the stunning result that the binding of the E79Q mutant to one side of the bilayer is no longer influenced by the presence of a bound mutant monobody on the opposite side of the bilayer. In other words, an acidic residue at position 79 is not only required to knock-off the monobody by voltage, but is also required for the negative cooperativity observed earlier. To strengthen these conclusions, Turman and Stockbridge (2017) look at the L2 monobody, which lacks an acidic residue at position 79, and see that this variant does not exhibit voltage-dependent dissociation and displays no evidence of negative cooperativity between toxin binding from opposite sides of the membrane. Finally, to complete the story, Turman and Stockbridge (2017) test whether the lifetime of the L3–Bpe complex is sensitive to the concentration of fluoride on the opposite side of the membrane and find that increasing the concentration of permeant ions promotes dissociation of WT L3 but not that of the E79Q mutant. Overall, the results compellingly support the conclusion that monobody inhibition occurs via a pore block mechanism.

The picture emerging from this work is one in which the mechanisms of inhibition of a naturally occurring scorpion toxin and a synthetic monobody have deep and unexpected similarities. In the case of the cation-selective BK channel, the toxin inserts a basic residue (K27) into the pore that can sense the presence of cations permeating from the opposite side, and in the case of L3 and the Bpe Flu channel, the monobody inserts an acidic residue (E79) into the pore to sense permeating anions. The most decisive new result supporting a pore-blocking mechanism of inhibition for L3 is that the E79Q mutant binds with equal affinity and kinetics to one side of the Flu channel regardless of whether a mutant monobody is bound to the opposite side. This result is a clear indication that the monobody does not influence the structure of Bpe and therefore that the way the monobody seals off the permeation pathways in the structure speaks directly to the mechanism of inhibition. Overall, this is a wonderful piece of detective work that provides deep insight into the mechanism of monobody inhibition of Flu channels and brings us back to the now classic studies on charybdotoxin block of potassium channels. It is fascinating to learn that the mechanism used by a scorpion toxin to block a potassium channel, which arose over the course of evolution, can be recapitulated with monobodies synthesized in the laboratory and selected for based only on their ability to bind Flu channels.

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