Gating on the outside

Andrew L. Harris

Department of Pharmacology and Physiology, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103

Connexin protein can form single-membrane spanning channels (called hemichannels or connexons) that can dock with each other to span two closely apposed plasma membranes and thereby create gap junction channels. What keeps unapposed connexin hemichannels closed, and how do they open when part of junctional channels? The report from Tang et al. (2009) in this issue (see p. 555), along with a recent publication from several of the same authors, goes a long way toward answering the first question and allows more specific questions to be asked regarding the second. The recently published crystal structure of a connexin channel informs both.

A hemichannel is a hexamer of connexin protein. Each connexin monomer has four transmembrane domains (TM1–TM4) with the amino and carboxyl termini cytoplasmic. The extracellular aspect of the hemichannel is composed of two extracellular loops (E1 and E2) from each connexin monomer.

Unapposed hemichannels are able to open under certain conditions but must remain closed most of the time to maintain the plasma membrane permeability barrier. The gating mechanism that achieves this is voltage sensitive and requires extracellular calcium ion in the millimolar range to remain closed at normal resting potentials (Ebihara and Steiner, 1993; Trexler et al., 2000; Ebihara et al., 2003; Puljung et al., 2004; Verselis and Srinivas, 2008).

This mechanism has acquired the eponym "loop gating" because of the composition of the extracellular domains, the effect of extracellular but not intracellular calcium ion on its function, and because at the single-channel level, the gating transitions resemble those that occur when a junctional channel forms (i.e., when the loop domains from apposed hemichannels interact) (Trexler et al., 1996). These transitions are distinct from those seen in other types of connexin channel gating.

In addition to remaining mostly closed when unapposed, the loop gate must also be able to open when docked to another hemichannel in the junctional configuration. The relationship between loop gating and the mechanism/structure of hemichannel docking is unclear; the two processes likely involve the same or adjacent portions of the protein, which raises intriguing structural and biophysical issues as to how the structural transitions are achieved.

Accessibility studies have shown that the physical loop gate and the site of calcium ion action that keep a hemichannel closed are both extracellular to residue 35 in TM1 (considered to span residues ~21–42) (Pfahnl and Dahl, 1999). This residue remains accessible from the cytoplasmic face of the channel when the loop gate is closed, indicating that the channel is occluded only extracellular to residue 35, and not along the whole length of the pore. For these reasons, the loop-gating mechanism is regarded as readily accessible from the extracellular space and presumed to be near the extracellular end of the channel.

A long-awaited high-resolution structure of a connexin channel was published recently (Maeda et al., 2009). The crystal structure does not identify the loop gate, as it is of an open channel. However, it does provide relevant information.

In this issue, Tang et al. (2009) use single-channel and macroscopic current recordings, cysteine mutagenesis, MTS and thiol cross-linking reagent accessibility and state-dependent studies of metal binding sites to investigate the conformational changes that occur with the loop-gating transition. The data yield information regarding pore-lining residues and the nature of the interconnexin interactions that result in a closed loop gate. This work follows another paper published recently that addresses the same issue employing many of the same techniques, using a different connexin (Verselis et al., 2009). The results from the two studies are, intriguingly, both similar and dissimilar.

Tang et al. worked with a chimeric Cx32 that readily opens as an unapposed hemichannel, enabling accessibility studies. They show that V38C and G45C are accessible to MTSEA–biotin-X (MTS-BX) from both sides of open hemichannels, reducing the single-channel conductance in stepwise fashion, indicating sequential modification of individual connexins. These residues are located in the extracellular half of the first transmembrane domain (TM1) and the contiguous first extracellular

Correspondence to Andrew L. Harris: aharris@umdnj.edu

The Rockefeller University Press $30.00
J. Gen. Physiol. Vol. 133 No. 6 549–553
www.jgp.org/cgi/doi/10.1085/jgp.200910256

loop (E1), respectively. Reaction with MTS-BX leaves the gating mechanisms intact. No reactivity was seen with cysteine substitution at the nearby positions V37, A39, A40, or A43 (Fig. 1).

Tang et al. show that loop gate closure corresponds to A43C residues from different monomers coming into close proximity and forming metal binding sites. Notably, this residue was not accessible to MTS-BX modification when the loop gate was open. There is similar evidence for close proximity of A40C residues during loop gating. It appears that the dominant interaction between the A43C residues and between A40C residues is the formation of metal binding sites, with lesser contribution from disulfide bond formation. These results are well-supported by several types of complementary experiments.

One particularly elegant feature is the use of a mutant to unambiguously assign the observed gating reaction to the loop gate. There are two voltage-sensitive gating mechanisms in these channels, both of which close in response to inside-negative voltages, with only the loop gate sensitive to extracellular calcium ion. Tang et al. introduced a point mutation in the amino-terminal domain that reverses the polarity to which the other gating mechanism ("Vj gate") closes. This allowed them to eliminate its participation in channel closure and show that in the absence of Vj gating, the A43C mutant forms a metal binding site when only loop gating was activated.

The two inferences drawn from these results, before publication of the crystal structure, were that the loop gate is located near the TM1/E1 border, and the loop gate is created by a substantial rotation of the TM1 helices and an inward tilt, allowing the A43 residues to rotate into the pore lumen and come into close proximity.

Recently published work on Cx50 hemichannels (Verselis et al., 2009) pointed to the same region as involved in loop gating, but with an important difference. That study found that F43/42[1] was both exposed to the pore in the open state and formed a metal binding site with loop gate closure. The fundamental difference with Tang et al. is that no rotation is required in the closing of the loop gate because F43/42 is accessible when the gate is open. Consistent with Tang et al., Verselis et al. also showed that G46/45 was accessible within in the pore, as well as D51/50.

The differences between the two sets of data suggested that perhaps the mechanism of loop gate formation is different in these two connexin isoforms. Connexins can be grouped into several families based on gene and protein sequences (Cruciani and Mikalsen, 2006). Cx32 is a member of one major group ("beta" connexins), as is Cx26, from which the crystal structure is derived. Cx50 is a member of the other major group ("alpha" connexins). Tang et al. used a chimera in which the first extracellular loop of Cx32 is replaced by that of Cx43, an "alpha" connexin, which greatly enhances the open probability of the hemichannel.

Even though the inferred gating mechanisms seem very different—no rotation versus a substantial rotation—the fact is that the two residues Tang et al. found to contribute to the gate bracket the residue that Verselis et al. identified as involved (A40 and A43 vs. F43/42). Significantly, the suggestion of TM1 rotation presumed α-helical structure in this region.

What does the crystal structure of Cx26 tell us about loop gating? Because the structure is of two open and apparently docked hemichannels, it cannot tell us what the closed loop gate looks like. It is, however, consistent with the findings of Tang et al. that A39, A40, and A43 are not exposed to the pore in the open state, and that G45 is. It is also consistent with the findings from Verselis et al. that G46/45 and D51/50 are accessible from the pore when open. Points of difference are that, unlike in the crystal structure, Tang et al. found V38 accessible from the open pore and V37 inaccessible, and Verselis et al. found F43/42 accessible.

The most important point from this aspect of the structure, however, is that TM1 does indeed line the pore of the open connexin channel, corresponding to inferences from single-channel accessibility and chimeric studies of hemichannels (Zhou et al., 1997; Kronengold et al., 2003; Oh et al., 2008). This finding contrasts with accessibility studies on junctional channels using macroscopic currents and the conclusions reached by studies based on lower resolution structures, modeling, and evolutionary inferences, suggesting that TM3 is the primary pore-lining helix (Skerrett et al., 2002; Fleishman et al., 2004; Pantano et al., 2008).

It is interesting to note that in the crystal the sites and structures of interactions between the hemichannels are quite different and much more restricted (and seemingly less robust) than those inferred from previous work (Foote et al., 1998). A skeptic could assert that this difference raises the possibility that the docking of the hemichannels in the crystal may not be of the same character as that which occurs in true junctional channels; the starting material for the crystal was single hemichannels, which "docked" during crystallization. Nevertheless, because TM3 is on the periphery of the channel in the crystal structure, its movement to be the primary pore-lining helix as a consequence of docking seems unlikely, even if the docking interactions in the crystal are not biologically accurate.

Regarding the loop gate, the crystal structure places the residues surrounding the TM1-E1 transition in the

[1]Residues in Cx50 are displaced by a single position from those in the two other connexins discussed. In this article, the Cx50 residue number is followed by a forward slash and the corresponding position in Cx26 and Cx32.

Figure 1. Alignment of amino acid segments involved in formation of the loop gate. The relevant segments from the three connexins studied in the discussed papers are shown. Cx32*Cx43E1 is the form studied in Tang et al. in this issue. Cx50 is the form studied by Verselis et al. Cx26 is the form that was used in the crystal structure of Maeda et al. By convention, residues 21–42 compose TM1 and 43–70 compose E1, so the segments above correspond to the extracellular portion of TM1 and a contiguous portion of E1. Accessibility referred to below is based on thiol reactivity and/or formation of metal binding sites when the indicated residues are replaced by cysteines. Red, residues accessible/reactive from both sides of the pore when loop gate is open; green, residues not accessible/reactive when the loop gate is open but forms metal binding site and/or can be cross-linked by dibromobimane when loop gate is closed; purple, residues that show no accessibility/reactivity under any tested conditions; pink, residues accessible/reactive when loop gate is open and when closed; blue highlight, residues identified as pore lining in the Maeda et al. crystal structure; box, 3_{10} helix identified in the Maeda et al. crystal structure.

appropriate position to form a gate (i.e., lining the pore), so it must be considered strong support for the direct involvement of this region in loop gating. As for the relatively minor differences in the specific residues involved and exposed to the lumen in the three studies, one can appeal to the different connexins used in each study and the differences between functioning hemichannels in plasma membrane and in associated/docked hemichannels in a crystal.

In addition, the crystal structure suggests a more specific and testable explanation for the mechanism of loop gate formation and the differences between the Tang et al. and Verselis et al. studies. An unexpected finding in the crystal structure was the presence of a short 3_{10} helix starting at the extracellular end of TM1 extending into E1, comprising residues V43 through E47 of Cx26. This means that the substantial rotation of TM1 originally postulated by Tang et al. to account for the lack of A43C accessibility in the open pore may not be required; instead, flexibility at or near residue 43 could effect the required changes in accessibility and formation of metal binding sites, as pointed out in the Appendix that accompanies the article. The rotation had been suggested assuming that the α-helical nature of TM1 extended through A43, but because this is not the case in the crystal structure, a less extreme conformational change would be required. This means that the mechanism of loop gating could be quite similar in the two connexins. One awaits computational and biophysical studies to explore this idea.

Now that it seems clear that TM1 lines the pore in this region and that loop gating occurs at the TM1-E1 transition region, what are the implications for the structural transitions that occur with hemichannel docking? One possibility is that the two processes are tightly linked—the binding of one hemichannel to another requires that the loop gate de-occlude the lumen. A potential triggering step is that the close approach of the extracellular domains of the hemichannels displaces postulated calcium ion(s) that keeps the extracellular end sealed by the loop gate. In this scenario, docking and opening of the loop gate occur simultaneously as part of the same concerted conformational change. The loop gate and the docking structure would be allosterically and conformationally linked, but the specific residues that form the gate and that interact directly with the apposed hemichannel need not be the same.

An alternative possibility is that the binding and the opening are two distinct processes that are not obligatorily linked. This scenario permits docking without opening of the pore; docking would enable opening but not require it. In this case, the structural elements involved in the two processes would need to be distinct.

Some connexin mutations allow docking but not channel opening (Hülser et al., 2001; Beahm et al., 2006), but the absence of opening cannot necessarily be attributed to the loop gate remaining closed. There have been reports of conductance transitions in junctional channels that resemble loop-gating transitions, but it is difficult to assign them unequivocally to this mechanism (Oh et al., 1997).

If the crystal structure does portray a physiological docking interaction, it suggests that the residues and segments of E1 involved in the inter-hemichannel contacts are six residues distal to those identified as involved in the loop gate in Tang et al. and Verselis et al. (see Fig. 2 and Fig. S3 of Maeda et al., 2009). It is possible that formation of the inter-hemichannel contacts by E1 pulls residues 40–44 out of the lumen, disrupting the gate. The crystal structure suggests substantial intra- and inter-protomer interactions in this region.

Alternatively, the residues identified by Tang et al. and Verselis et al. as coming into close proximity during loop gating may not be the only ones to do so and may be only part of the loop gate. That is, other, more distal portions of E1 could also directly participate in loop gate formation, in which case some of the residues that form the gate could also mediate docking.

What about the basis of the voltage sensitivity of the loop gate? This is not addressed by the studies under consideration. As mentioned above, connexin channels

have at least one other gating mechanism, known as Vj gating, which closes the channels to a substate and is well characterized at the single-channel level. The domains and the kinds of motion involved have been inferred from detailed biophysical studies (Oh et al., 2000, 2004; Purnick et al. 2000a,b; Srinivas et al., 2005; Oshima et al., 2007). It was established long ago that the sensor for this type of gating was within the pore, rather than extrinsic to it (Spray et al., 1981; Harris et al., 1981; Verselis et al., 1994; Oh et al., 2000). The recent crystal structure supports this and suggests a specific set of structural rearrangements that occur to effect Vj gating, largely consistent with the previous biophysical data. It appears that the sensor/gate is composed of the amino-terminal domain of the protein, which when the gate is open is folded into the lumen of pore against TM1, forming the pore wall in the cytoplasmic end of the pore. The suggestion is that in response to an appropriate electric field, these domains peel off the pore wall and move toward the cytoplasm to collapse into an aggregate that largely occludes the lumen. This is a unique voltage-dependent gating mechanism (connexin does not contain an S4-like domain), operating at the opposite end of the pore from the loop gate.

Detailed studies of interactions between Vj gating and loop gating in single channels suggest that the operation of the two gates is contingent (Bukauskas et al., 2001). Specifically, the data suggest that the voltage sensors of the two mechanisms are in series in the lumen of the pore, and that the sensitivity of each to applied voltage changes with the position of the other gate. This is reminiscent of the mechanism proposed for contingent operation of macroscopic Vj gates in each hemichannel of a junctional channel (Harris et al., 1981).

Accessibility-based studies of connexin channel gating are just beginning. The availability of an initial high-resolution structure of a connexin channel is a most welcome development. As is the case for any first high-resolution structure, there is substantial excitement as predictions from biophysical studies are brought into structural focus and as new questions arise. As for other channels, structural inferences from biophysical studies have turned out to be reasonably accurate and provide insight to the new structural information. Also as true for other channels, the crystal provides unanticipated structural information that must be incorporated and accounted for in the biophysical work that preceded and follows it. This is a welcome challenge.

REFERENCES

Beahm, D.L., A. Oshima, G.M. Gaietta, G.M. Hand, A.E. Smock, S.N. Zucker, M.M. Toloue, A. Chandrasekhar, B.J. Nicholson, and G.E. Sosinsky. 2006. Mutation of a conserved threonine in the third transmembrane helix of alpha- and beta-connexins creates a dominant-negative closed gap junction channel. *J. Biol. Chem.* 281:7994–8009.

Bukauskas, F.F., Q. Shao, A. Bukauskiene, M.V.L. Bennett, D.W. Laird, and V.K. Verselis. 2001. Gating properties of gap junction channels assembled from connexin43 and connexin43 fused with green fluorescent protein. *Biophys. J.* 81:137–152.

Cruciani, V., and S.O. Mikalsen. 2006. The vertebrate connexin family. *Cell. Mol. Life Sci.* 63:1125–1140.

Ebihara, L., and E. Steiner. 1993. Properties of a nonjunctional current expressed from a rat connexin46 cDNA in Xenopus oocytes. *J. Gen. Physiol.* 102:59–74.

Ebihara, L., X. Liu, and J.D. Pal. 2003. Effect of external magnesium and calcium on human connexin46 hemichannels. *Biophys. J.* 84:277–286.

Fleishman, S.J., V.M. Unger, M. Yeager, and N. Ben-Tal. 2004. A C-alpha model for the transmembrane alpha helices of gap junction intercellular channels. *Mol. Cell.* 15:879–888.

Foote, C.I., L. Zhou, X. Zhu, and B.J. Nicholson. 1998. The pattern of disulfide linkages in the extracellular loop regions of connexin32 suggests a model for the docking interface of gap junctions. *J. Cell Biol.* 140:1187–1197.

Harris, A.L., D.C. Spray, and M.V.L. Bennett. 1981. Kinetic properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* 77:95–117.

Hülser, D.F., M.L. Rutz, R. Eckert, and O. Traub. 2001. Functional rescue of defective mutant connexons by pairing with wild-type connexons. *Pflugers Arch.* 441:521–528.

Kronengold, J., E.B. Trexler, F.F. Bukauskas, T.A. Bargiello, and V.K. Verselis. 2003. Single-channel SCAM identified pore-lining residues in the first extracellular loop and first transmembrane domains of Cx46 hemichannels. *J. Gen. Physiol.* 122:389–405.

Maeda, S., S. Nakagawa, M. Suga, E. Yamashita, A. Oshima, Y. Fujiyoshi, and T. Tsukihara. 2009. Structure of the connexin 26 gap junction channel at 3.5 A resolution. *Nature.* 458:597–602.

Oh, S., Y. Ri, M.V.L. Bennett, E.B. Trexler, V.K. Verselis, and T.A. Bargiello. 1997. Changes in permeability caused by connexin32 mutations underlie X-linked Charcot-Marie-Tooth disease. *Neuron.* 19:927–938.

Oh, S., C.K. Abrams, V.K. Verselis, and T.A. Bargiello. 2000. Stoichiometry of transjunctional voltage-gating polarity reversal by a negative charge substitution in the amino terminus of a connexin32 chimera. *J. Gen. Physiol.* 116:13–31.

Oh, S., S. Rivkin, Q. Tang, V.K. Verselis, and T.A. Bargiello. 2004. Determinants of gating polarity of a connexin 32 hemichannel. *Biophys. J.* 87:912–928.

Oh, S., V.K. Verselis, and T.A. Bargiello. 2008. Charges dispersed over the permeation pathway determine the charge selectivity and conductance of a Cx32 chimeric hemichannel. *J. Physiol.* 586:2445–2461.

Oshima, A., K. Tani, Y. Hiroaki, Y. Fujiyoshi, and G.E. Sosinsky. 2007. Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule. *Proc. Natl. Acad. Sci. USA.* 104:10034–10039.

Pantano, S., F. Zonta, and F. Mammano. 2008. A fully atomistic model of the Cx32 connexon. *PLoS One.* 3:e2614.

Pfahnl, A., and G. Dahl. 1999. Gating of Cx46 gap junction hemichannels by calcium and voltage. *Pflugers Arch.* 437:345–353.

Puljung, M.C., V.M. Berthoud, E.C. Beyer, and D.A. Hanck. 2004. Polyvalent cations constitute the voltage gating particle in human connexin37 hemichannels. *J. Gen. Physiol.* 124:587–603.

Purnick, P.E.M., D.C. Benjamin, V.K. Verselis, T.A. Bargiello, and T.L. Dowd. 2000a. Structure of the amino terminus of a gap junction protein. *Arch. Biochem. Biophys.* 381:181–190.

Purnick, P.E.M., S.H. Oh, C.K. Abrams, V.K. Verselis, and T.A. Bargiello. 2000b. Reversal of the gating polarity of gap junctions by negative charge substitutions in the N-terminus of connexin 32. *Biophys. J.* 79:2403–2415.

Skerrett, I.M., J. Aronowitz, J.H. Shin, G. Cymes, E. Kasperek, F.L. Cao, and B.J. Nicholson. 2002. Identification of amino acid residues lining the pore of a gap junction channel. *J. Cell Biol.* 159:349–359.

Spray, D.C., A.L. Harris, and M.V.L. Bennett. 1981. Equilibrium properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* 77:77–93.

Srinivas, M., J. Kronengold, F.F. Bukauskas, T.A. Bargiello, and V.K. Verselis. 2005. Correlative studies of gating in Cx46 and Cx50 hemichannels and gap junction channels. *Biophys. J.* 88:1725–1739.

Tang, Q., T.L. Dowd, V.K. Verselis, and T.A. Bargiello. 2009. Conformational changes in a pore-forming region underlie voltage-dependent "loop gating" of an unapposed connexin hemichannel. *J. Gen. Physiol.* 133:555–570.

Trexler, E.B., M.V.L. Bennett, T.A. Bargiello, and V.K. Verselis. 1996. Voltage gating and permeation in a gap junction hemichannel. *Proc. Natl. Acad. Sci. USA.* 93:5836–5841.

Trexler, E.B., F.F. Bukauskas, J. Kronengold, T.A. Bargiello, and V.K. Verselis. 2000. The first extracellular loop domain is a major determinant of charge selectivity in connexin46 channels. *Biophys. J.* 79:3036–3051.

Verselis, V.K., and M. Srinivas. 2008. Divalent cations regulate connexin hemichannels by modulating intrinsic voltage-dependent gating. *J. Gen. Physiol.* 132:315–327.

Verselis, V.K., C.S. Ginter, and T.A. Bargiello. 1994. Opposite voltage gating polarities of two closely related connexins. *Nature.* 368:348–351.

Verselis, V.K., M.P. Trelles, C. Rubinos, T.A. Bargiello, and M. Srinivas. 2009. Loop gating of connexin hemichannels involves movement of pore-lining residues in the first extracellular loop domain. *J. Biol. Chem.* 284:4484–4493.

Zhou, X.W., A. Pfahnl, R. Werner, A. Hudder, A. Llanes, A. Luebke, and G. Dahl. 1997. Identification of a pore lining segment in gap junction hemichannels. *Biophys. J.* 72:1946–1953.