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COMMENTARY

Unconventional transport of metal ions and protons by Nramps

Gary Rudnick

Nramps are divalent transition metal ion transporters found in essentially all organisms from prokaryotes to mammals (Nevo and Nelson, 2006). The name Nramp comes from natural resistance-associated macrophage protein, because it was first identified as a protein that conferred resistance to mycobacteria in mice (Vidal et al., 1993). The cloned gene contained regions that looked like they would form transmembrane helical regions, resulting in the suggestion that it was a transport protein (Supek et al., 1996). It is now well established that Nramps serve to transport metals such as Mn^{2+} and Fe^{2+}. They represent a family of transporters with similar sequences, found in mammals, yeast, flies, and bacteria, which have been classified as the SLC11 family. In 2014, the first structure of an Nramp transporter was published (Ehrnstorfer et al., 2014). This structure established that the SLC11 family belonged to the APC/FIRL superfamily of transporters, all of which have the characteristic fold of LeuT (Khafizov et al., 2012; Vastermark et al., 2014). In this issue of the *Journal of General Physiology*, Bozzi et al. present an extensive kinetic study that highlights the diversity of mechanisms within this large superfamily of transport proteins.

Despite having the same fold, the individual transporter subfamilies in the APC/FIRL superfamily vary widely in their ion substrate–coupling mechanisms. Some are ion-independent exchangers (Fang et al., 2009), some are tightly coupled Na^+ symporters (Radian and Kanner, 1983; Roux and Supplisson, 2000), and at least one subfamily comprises proton antiporters (McIntire et al., 1997). Even within the Na^+ symporter subfamilies, for example, there are significant differences in the role that Na^+ plays in conformational change (Kazmier et al., 2014). More than ever, we are being forced to think of the LeuT fold not as defining a specific mechanism for substrate transport, but rather as a framework with the flexibility to accommodate multiple conformational and coupling mechanisms.

For most of the transporters that have been studied in this superfamily, the conformational mechanism of transport is relatively conventional. Substrates and coupling ions move through the membrane by a process in which a central binding site is alternately exposed to one side of the membrane or the other (Shimamura et al., 2010; Krishnamurthy and Gouaux, 2012; Perez et al., 2012). Permeation pathways open and close in a coordinated manner to facilitate efficient substrate and ion movement (Fig. 1). These pathway openings and closings result from reorientation of a "bundle" or "core" domain, consisting of four transmembrane (TM) helices, within a scaffold domain consisting of the remaining TM helices (Forrest, 2015).

The Nramps have been described as proton–metal symporters (Gunshin et al., 1997). As such, the influx of metals should be driven by the pH difference (ΔpH) that exists between the cytoplasm and the extracellular medium in prokaryotes, plants, and fungi. ΔpH should also drive metal efflux from acidic organelles such as lysosomes and phagosomes, which use Nramps to decrease the content of lumenal metals. Because they move positive charges across the membrane, a transmembrane electrical potential (Δψ) also serves as a force that drives metal ions and protons toward the relatively negative side of the membrane. For example, the inside negative potential in mammalian and bacterial cells should drive metal uptake through Nramps. However, the current work indicates that coupling between proton and substrate transport is variable, depending not only on conditions, as observed for other Nramps (Chen et al., 1999), but also on the metal ion substrate. Although Δψ and ΔpH are thermodynamic driving forces, they can also dramatically affect transport kinetics in a metal-dependent manner. Most unexpectedly, these new studies provide evidence that metal ions and protons may use separate transport pathways: metal ions traveling through the conventional pathway created by bundle movement, and protons traveling through a unique pathway within the scaffold domain.

Previously, the Gaudet laboratory had determined structures of the Nramp homologue from *Deinococcus radiodurans* (Dra) in several conformations (Bozzi et al., 2019b). They observed a unique feature of the structure: a series of polar residues and ion

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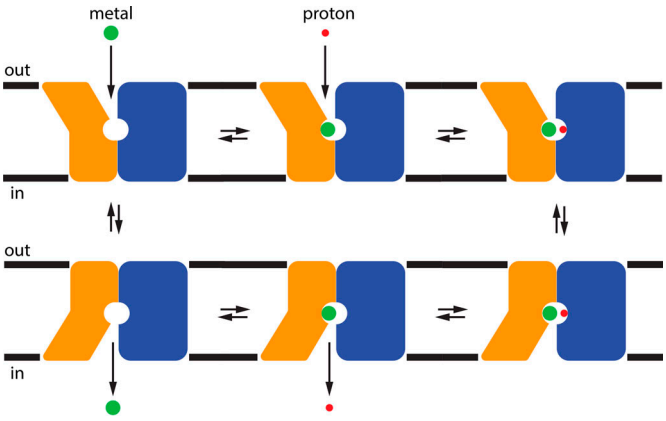
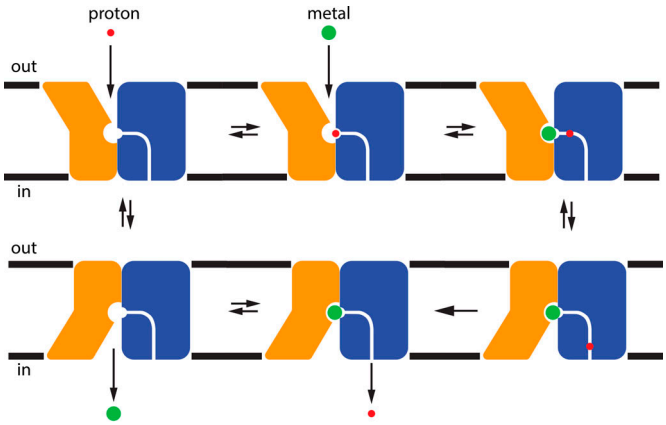


Canonical metal-proton symport

Figure 1. **A canonical mechanism for metal-proton symport.** In a conventional transport mechanism, metal and protons bind to central binding sites in the transporter in an outward-facing conformation. In APC/FIRL transporters, binding sites are found near the interface between the scaffold (blue) and bundle (orange) domains. After binding metal (green) and proton(s) (red) to the outward-facing transporter (top), reorganization of the bundle domain (right) closes the extracellular pathway and opens a pathway between the binding sites and the cytoplasm (lower right). After release of metal and proton(s) to the cytoplasm (bottom), the bundle domain isomerizes (left), returning to the outward-facing conformation (top left).



Metal and proton flux through separate pathways

Figure 2. **Transport by a mixed mechanism with a proton channel.** In a mixed mechanism, metal (green) is transported by protein conformational changes as in the canonical mechanism (Fig. 1), but protons (red) can cross the membrane through a channel in the scaffold domain (blue). This mechanism could involve protonation of a residue in the binding site that coordinates the metal ion so that binding of the metal (top center) would displace the proton, injecting it into the channel (top right). Dissociation of the proton from the channel into the cytoplasm (bottom center) could be a difficult-to-reverse step, thereby minimizing the reverse reaction. How the transporter couples dissociation of protons and metal ions to the cytoplasm is unclear at present.

pairs that occur only in the Nramp family. Two ion pairs in the scaffold domain between TM3 and TM9, along with an aspartate, a glutamate, and a histamine in TM3 and TM6, provide a potential pathway for protons from the metal binding site through the scaffold domain to the cytoplasm. Dra Nramp, reconstituted into proteoliposomes, conducted a proton flux driven by $\Delta\psi$ (interior negative). This proton flux was eliminated when the transporter was locked in an inward-facing conformation by covalent modification, but Dra Nramp covalently locked in an outward-facing conformation still catalyzed proton flux. These findings were consistent with movement of protons through the open extracellular pathway and then through the ion pairs in the scaffold domain that form a pathway between the metal binding site and the cytoplasm. Mutation of any of the four residues connecting these ion pairs with the metal site blocked proton flux, supporting the proposal that these ion pairs formed a pathway for protons through the transporter.

Uncoupled ion flux has been observed in several otherwise coupled transporters. Within the APC/FIRL superfamily, biogenic amine transporters in the SLC6 family all catalyze uncoupled currents (Mager et al., 1994; Galli et al., 1996; Sonders et al., 1997). Excitatory amino acid transporters (EAATs) also have this property (Fairman et al., 1995). However, in those transporters the uncoupled flux was never shown to be coupled directly to substrate transport. In the EAATs the uncoupled conductance carries Cl^- ions, which are not required for ion-coupled substrate transport (Fairman et al., 1995).

In their latest contribution, Bozzi et al. (2019a) used Dra Nramp expressed in *Escherichia coli* and studied its function in intact cells and proteoliposomes. Metal influx was measured using Fura-2–loaded vesicles, and proton influx was measured from quenching of intravesicular BCECF. For experimental reasons, initial rates of metal or proton transport were easier to measure than equilibrium accumulation levels. These rates varied dramatically depending on the driving forces. Rates of metal uptake were strongly dependent on $\Delta\psi$, with higher rates associated with more negative inside potentials. Surprisingly, however, the response varied according to the metal. Cd^{2+} was the only metal tested that was appreciably transported in the absence of a potential ($\Delta\psi = 0$). Mn^{2+} was not transported in the absence of a potential, but influx increased as the interior was made more negative ($\Delta\psi < 0$). Transport of Fe^{2+} and Zn^{2+} also occurred only at negative values of $\Delta\psi$, but they required potentials more negative than −40 mV. Co^{2+} was transported only at values of $\Delta\psi$ less than −70 mV. Clearly, the nature of the metal ion bound to Dra Nramp influenced the kinetics of transport, suggesting the involvement of a rate-determining, voltage-dependent transition in the catalytic cycle. The variability with different metals suggests that this transition occurs while a metal ion substrate is bound to the transporter.

For both Mn^{2+} and Cd^{2+}, ΔpH (interior alkaline) increased the rate of metal uptake. This was not simply the result of a pH-dependent step on one side of the membrane because influx was increased both by increasing internal pH and by decreasing external pH and was decreased by reversing the ΔpH (interior acid). These data show that both ΔpH and $\Delta\psi$ can influence the rate of metal transport and suggest that the rate-determining step in transport involves inward movement of a proton across the membrane. It is tempting to extrapolate from these effects of ΔpH and $\Delta\psi$ on transport rate that Dra Nramp stoichiometrically couples metal and proton movements. However, these rate measurements tell us only about the rate-determining

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step in transport and not the overall reaction. Comparing rates of metal and proton transport showed that, although the rate of Mn^{2+} and Cd^{2+} transport responded similarly to ΔpH, they differed greatly in their ability to stimulate proton influx.

By measuring proton and metal influx under the same conditions, the relative rates could be used to estimate a stoichiometry of metal-proton symport. Surprisingly, the relative flux ratios varied widely depending on the metal substrate. At −80 mV, where basal proton influx is minimal in the absence of metal, Cd^{2+} was taken up readily with little effect on H^+ influx. Mn^{2+} transport, however, was accompanied by H^+ influx at a ratio of ∼1:1. For Fe^{2+}, it was 3:1, and influx of other metals was low. At −120 mV, where significant H^+ flux was observed in the absence of metal, Cd^{2+} decreased basal H^+ influx, but both Mn^{2+} and Fe^{2+} increased proton influx, as they did at −80 mV. The influx of Mn^{2+} and Fe^{2+} was consistently associated with proton entry, but with different ratios of metal ions to protons, and Cd^{2+} influx apparently suppressed basal H^+ entry rather than stimulating it. These results indicated markedly different modes of H^+-metal coupling, depending on the metal ion substrate.

These results raise a question: Is the transport of metal and proton(s) coupled stoichiometrically? And if so, does the metal ion move together with the proton in the conventional way that an amino acid or a sugar might be transported with a Na^+ ion in other structurally related APC/FIRL family members? The alternative is that there is stoichiometric coupling between metal transported by conventional conformational changes and protons transported through the network of ion pairs (Fig. 2). Indeed, previous work identified four residues that were essential for uncoupled proton flux through Dra Nramp, and mutating any of those four residues perturbed metal-stimulated proton flux.

If the metal and the proton travel through different pathways, it is not obvious how their movement could be stoichiometrically coupled. One possibility, proposed by Bozzi et al. (2019a), is that the proton could be injected into the pathway by metal ion binding. An aspartate in the metal site has the potential to lose a proton upon binding of some metals, such as Mn^{2+}, that have an incomplete shell of d-electrons. Alternatively, binding of another metal, such as Cd^{2+}, which has a full d-shell, would not displace the proton. Another possibility is that proton transport through the ion pair network could somehow initiate the conformational changes responsible for metal transport. The observation that Dra Nramp locked in an outward-facing conformation still allows uncoupled proton flux argues against such coupling between proton flux and conformational change. In fact, metal and proton transport might not be coupled directly. Different metals might simply stimulate or inhibit the basal proton flux to varying degrees, leading to a greater or lesser number of protons, on average, crossing the membrane per metal ion. Answers to these questions will require additional study of the transporter thermodynamics.

Asking why nature has chosen such an unusual mechanism for the Nramps may be an exercise in frustration, but it is still tempting to speculate. One possibility proposed by Bozzi et al. (2019a) is that this mechanism may ensure that Nramp is very unlikely to catalyze efflux of the accumulated metals. To test this hypothesis, they generated proteoliposomes with functional Dra Nramp in the everted orientation, i.e., with the extracellular face of the protein facing the vesicle lumen. Wild type Dra Nramp in this inside-out orientation is incapable of accumulating Mn^{2+} (corresponding to efflux in right-side out vesicles). However, mutants in the ion pair network have a markedly increased ability to efflux Mn^{2+} into the proteoliposomes. This may be a sign that the unusual coupling between metal and proton fluxes ensures that a steep kinetic barrier prevents the reversal of this metabolically important reaction.

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