Crystal Structure and Conformational Change Mechanism of a Bacterial Nramp-Family Divalent Metal Transporter

Graphical Abstract

**Highlights**
- *Deinococcus radiodurans* Nramp structure reveals TM1a location in inward-open state
- Unfettered movement of TM1a is essential to the metal-transport cycle
- G153R disease-mutant mimic alters selectivity of conserved metal-binding site
- G45R disease-mutant mimic sterically locks protein in inward-open state

**Authors**
Aaron T. Bozzi, Lukas B. Bane, Wilhelm A. Weihofen, ..., Hidde L. Ploegh, Klaus Schulten, Rachelle Gaudet

**Correspondence**
gaudet@mcb.harvard.edu

**In Brief**
Bozzi et al. determined the inward-open structure of a bacterial Nramp transition metal transporter with a LeuT fold. Using biochemical experiments, the authors provide mechanistic explanations for how two anemia-causing mutations impede function through altering the conformational landscape of the protein in unique ways.

**Accession Numbers**
5KTE

Bozzi et al., 2016, *Structure* 24, 1–13
December 6, 2016 © 2016 Elsevier Ltd.
http://dx.doi.org/10.1016/j.str.2016.09.017
Crystal Structure and Conformational Change Mechanism of a Bacterial Nramp-Family Divalent Metal Transporter

Aaron T. Bozzi,1,5 Lukas B. Bane,1,5,6 Wilhelm A. Weihofen,1,5,7 Abhishek Singharoy,2 Eduardo R. Guillen,4,8 Hidde L. Ploegh,4 Klaus Schulten,2,3 and Rachelle Gaudet1,9,*

1Department of Molecular and Cellular Biology, Harvard University, 52 Oxford Street, Cambridge, MA 02138, USA
2Beckman Institute for Advanced Science and Technology
3Department of Physics
University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
4Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA
5Co-first author
6Present address: D.E. Shaw Research, New York, NY 10036, USA
7Present address: Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA
8Present address: EpiVax, Inc, 146 Clifford Street, Providence, RI 02903, USA
9Lead Contact
*Correspondence: gaudet@mcb.harvard.edu
http://dx.doi.org/10.1016/j.str.2016.09.017

SUMMARY

The widely conserved natural resistance-associated macrophage protein (Nramp) family of divalent metal transporters enables manganese import in bacteria and dietary iron uptake in mammals. We determined the crystal structure of the Deinococcus radiodurans Nramp homolog (DraNramp) in an inward-facing apo state, including the complete transmembrane (TM) segment 1a (absent from a previous Nramp structure). Mapping our cysteine accessibility scanning results onto this structure, we identified the metal-permeation pathway in the alternate outward-open conformation. We investigated the functional impact of two natural anemia-causing glycine-to-arginine mutations that impaired transition metal transport in both human Nramp2 and DraNramp. The TM4 G153R mutation perturbs the closing of the outward metal-permeation pathway and alters the selectivity of the conserved metal-binding site. In contrast, the TM1a G45R mutation prevents conformational change by sterically blocking the essential movement of that helix, thus locking the transporter in an inward-facing state.

INTRODUCTION

Nearly all organisms require iron to survive. The oxidation state cycle of iron is ideal both to catalyze essential redox reactions as a co-factor in numerous enzymes and to provide a pathway for electron transport across membranes. In addition, heme iron is used for oxygen transport and storage, enabling aerobic respiration. Organisms have thus evolved mechanisms to acquire, traffic, and safely store this crucial transition metal (Aisen et al., 2001; Andrews, 2008). The natural resistance-associated macrophage protein (Nramp) family of divalent metal transporters plays a vital role in mammalian iron homeostasis (Mackenzie and Hediger, 2004). Expressed in phagosomal membranes, Nramp1 both helps macrophages kill engulfed pathogens by extracting iron and other essential transition metals (Cellier et al., 2007), and enables iron recycling from dying erythrocytes (Soe-Lin et al., 2009). Mammals abundantly express a second homolog, Nramp2 (also known as divalent metal transporter 1) in intestinal enterocytes to enable absorption of dietary iron (Gunshin et al., 1997). Nramp2 is also expressed at lower levels in the endosomal membranes of most somatic cells (Mackenzie et al., 2007), where it enables extraction of transferrin-bound iron from vesicles, an especially important process in erythropoiesis (Canonne-Hergaux et al., 2001; Gunshin et al., 2005).

To maintain homeostasis, mammals tightly regulate iron uptake and transport (Lieu et al., 2001), primarily through translation and localization of Nramps (Gunshin et al., 2001; Hubert and Hentze, 2002). An overabundance of free iron generates free radicals that cause tissue damage and increase susceptibility to infection (Ganz, 2009). In contrast, iron deficiency causes anemia (Abbaspour et al., 2014). Accordingly, mutations in Nramp2 are implicated in anemia in humans and rodents (Iolascon and De Falco, 2009; Shawki et al., 2012). The same glycine-to-arginine mutation (G185R) causes microcytic anemia in both mice (Fleming et al., 1997) and Belgrade rats (Fleming et al., 1998; Veuthey and Wessling-Resnick, 2014), and altered protein localization in enterocytes (Canonne-Hergaux et al., 2000). This mutation reduces iron transport when expressed in mammalian cell lines (Su et al., 1998; Touret et al., 2004; Xu et al., 2004), with a concomitant increase in permeability to calcium (typically a poor Nramp substrate) in G185R-transfected cells compared with wild-type (WT) counterparts (Xu et al., 2004). A glycine-to-arginine mutation (G75R) in human anemia patients may abrogate iron transport function (Barrios et al., 2012; Bianco et al., 2009; Shawki et al., 2012). However, the molecular mechanisms
Table 1. Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th></th>
<th>Fab</th>
<th>DraNramp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB</td>
<td>NA</td>
<td>5KTE</td>
</tr>
<tr>
<td>SBGrid Databank</td>
<td>335</td>
<td>332, 333, 334</td>
</tr>
</tbody>
</table>

### Data Collection

<table>
<thead>
<tr>
<th></th>
<th>Fab</th>
<th>DraNramp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>0.97917</td>
<td>1.139</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>30.00–3.00</td>
<td>46.47–3.94</td>
</tr>
<tr>
<td>(3.11–3.00)</td>
<td>(4.08–3.94)</td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P2_12121</td>
<td>I222</td>
</tr>
<tr>
<td>Unit cell (a, b, c)</td>
<td>116.17, 183.66, 299.88</td>
<td>113.13, 132.08, 221.0</td>
</tr>
<tr>
<td>No. of crystals</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total reflections</td>
<td>558,353</td>
<td>245,867</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>119,718</td>
<td>11,791 (462)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.7 (4.8)</td>
<td>16.6 (11.4)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.5 (100.0)</td>
<td>95 (96)</td>
</tr>
<tr>
<td>Mean I/σ(I)</td>
<td>8.4 (2.2)</td>
<td>6 (6.1)</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.151</td>
<td>0.171</td>
</tr>
<tr>
<td>Rmeas</td>
<td>0.177</td>
<td>0.177</td>
</tr>
<tr>
<td>Rfree</td>
<td>0.2666 (0.2672)</td>
<td>0.3128 (0.3656)</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>46.47–3.94</td>
<td>46.47–3.94</td>
</tr>
<tr>
<td>(4.12–3.94)</td>
<td>(4.08–3.94)</td>
<td></td>
</tr>
<tr>
<td>Refinement</td>
<td>Fab</td>
<td>DraNramp</td>
</tr>
<tr>
<td>No. of atoms</td>
<td>5,622</td>
<td>5,625</td>
</tr>
<tr>
<td>Protein</td>
<td>5,622</td>
<td>3</td>
</tr>
<tr>
<td>Protein residues</td>
<td>762</td>
<td>762</td>
</tr>
<tr>
<td>Ramachandran plot (%)</td>
<td>Favored</td>
<td>692 (90.8)</td>
</tr>
<tr>
<td>Allowed</td>
<td>66 (8.7)</td>
<td>66 (8.7)</td>
</tr>
<tr>
<td>Outliers</td>
<td>4 (0.52)</td>
<td>4 (0.52)</td>
</tr>
<tr>
<td>RMSD (bonds)</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>RMSD (angles)</td>
<td>1.12</td>
<td>1.12</td>
</tr>
<tr>
<td>Average B factor</td>
<td>89.8</td>
<td>89.8</td>
</tr>
<tr>
<td>Protein</td>
<td>89.8</td>
<td>89.8</td>
</tr>
<tr>
<td>Ions (Os)</td>
<td>190</td>
<td>3</td>
</tr>
</tbody>
</table>

NA, not applicable; RMSD, root-mean-square deviation.

RESULTS

The DraNramp Inward-Facing Structure Shows a Highly Kinked Transmembrane Helix 1

We determined the crystal structure of detergent-solubilized DraNramp (38% sequence identity with ScaNramp) in complex with a monoclonal antibody fragment (Fab) to 3.9-Å resolution (Table 1). Crystallization was facilitated by intracellular surface mutations (Figure 1B; see below). We used a ScaNramp-based homology model and the Fab fragment crystal structure as a molecular replacement (MR) search models, with additional phasing provided by single-wavelength anomalous signals from three osmium ions bound to the Fab or at crystal contacts (Figure S1). Refinement was facilitated by using xMDD (McGreavy et al., 2014; Singhary et al., 2015). In particular, we used a combination of xMDD and steered molecular dynamics to optimize our TM1a model, exploring different TM1a positional registries corresponding to a screw axis rotation of approximately one helical turn. The assigned TM1a registry (Figure 1D) yielded both the lowest R factor and the best agreement with cysteine accessibility data (see Figures 6 and 7; see below). The final asymmetric unit comprises one DraNramp transporter, one Fab bound to the DraNramp periplasmic face, and three osmium ions, with crystal-packing interactions between the Fab and the DraNramp cytoplasmic face (Figure S1). All 11 DraNramp TMs are visible in the electron density, including TM1a, which was truncated in ScaNramp (Ehrnstorfer et al., 2014).

Like ScaNramp, DraNramp has a LeuT fold with two pseudo-symmetry-related structural repeats comprising TM1–5. Because of the alternating access model for membrane transport proteins (Jardetzky, 1966), Nramps should cycle between at least two stable conformations: outward-facing to bind its metal substrate, and inward-facing to release its cargo into the cytosol. The ScaNramp structure, in an inward-facing state, revealed a metal-binding site that consists of conserved aspartate, asparagine, and methionine residues, and a backbone carbonyl from transmembrane segments (TMs) 1 and 6 that coordinate a range of divalent metal substrates (Ehrnstorfer et al., 2014), with the methionine providing a selective preference for transition metals over alkaline earth metals (Bozzi et al., 2016).

Here we present the crystal structure of the Deinococcus radiodurans Nramp homolog (DraNramp) from prokaryotic clade A. The DraNramp structure represents an inward-facing apo conformation, with TM1a, absent from the ScaNramp crystallization construct, swung up to open a large intracellular vestibule. We use this structure along with extensive cysteine accessibility and metal-transport measurements to propose a model for conformational change in this LeuT-fold transporter and explain the mechanistic effects of two anemia-causing mammalian Nramp2 mutations. In our model, reaching the outward-facing state requires TM1a to approach the protein core to close the intracellular vestibule. This motion is prevented by an N-terminal glycine-to-arginine mutation that mimics an anemia-causing mutation in human Nramp2. The second disease-mimicking glycine-to-arginine mutation alters the extracellular vestibule and metal-binding site, resulting in reduced transport activity and altered selectivity.

The Nramp family spans the tree of life, with homologs that perform a range of essential divalent transition metal-transport functions, likely as secondary transporters that harness a proton gradient (Courville et al., 2006). Based on sequence analyses, Nramp homologs form four major phylogenetic clades: the eukaryotic Nramps and the prokaryotic A, B, and C clades (Cellier et al., 2001). The crystal structure of Staphylococcus capitis Nramp (ScaNramp), belonging to clade C, confirmed a LeuT fold (named for the bacterial sodium/amino acid symporter [Yamashita et al., 2005]) for Nramps, as predicted previously (Cellier, 2012; Ehrnstorfer et al., 2014).

by which these mutations perturb Nramp metal transport and cause anemia remain unknown.

The Nramp family spans the tree of life, with homologs that perform a range of essential divalent transition metal-transport functions, likely as secondary transporters that harness a proton gradient (Courville et al., 2006). Based on sequence analyses, Nramp homologs form four major phylogenetic clades: the eukaryotic Nramps and the prokaryotic A, B, and C clades (Cellier et al., 2001). The crystal structure of Staphylococcus capitis Nramp (ScaNramp), belonging to clade C, confirmed a LeuT fold (named for the bacterial sodium/amino acid symporter [Yamashita et al., 2005]) for Nramps, as predicted previously (Cellier, 2012; Ehrnstorfer et al., 2014).
and TMs 6–10, respectively (Figures 1A and 1B) (Yamashita et al., 2005). As in other LeuT folds, TMs 1, 2, 6, and 7 form a “rocking bundle” whose putative movements relative to the “scaffold,” made up of the remaining TMs, likely effects the switch in active site accessibility from extracellular to intracellular (Forrest and Rudnick, 2009). TM1 and 6 are unwound in the center of the membrane plane, providing substrate-binding residues as observed in ScaNramp (Ehrnstorfer et al., 2014). In DraNramp, the metal-binding site is unoccupied and exposed to the intracellular side (see Figure 2A). Thus the structure resembles a substrate-free inward-facing conformation. Accordingly, DraNramp and ScaNramp superimpose well with a root-mean-square distance of 1.54 Å over 279 Ca atoms. Clade A members such as DraNramp are bent outward and lies nearly parallel to the membrane plane, providing substrate-binding residues as observed in ScaNramp (Ehrnstorfer et al., 2014). In DraNramp, the metal-binding site is unoccupied and exposed to the intracellular side (see Figure 2A). Thus the structure represents a substrate-free inward-facing conformation. Accordingly, DraNramp and ScaNramp superimpose well with a root-mean-square distance of 1.54 Å over 279 Ca atoms. Clade A members such as DraNramp have a four-residue deletion near the TM9 N terminus in comparison with clade C members such as ScaNramp, resulting in a shorter helix (Figure S1D) (Cellier, 2012). The main difference between the two structures is the position of scaffold helix TM5 (Figure 1C), more angled relative to the membrane plane in DraNramp, perhaps influenced by the presence of TM1a in the structure.

As in other LeuT-fold proteins (Figure 1E), DraNramp TM1a is bent outward and lies nearly parallel to the membrane plane, providing substrate-binding residues as observed in ScaNramp (Ehrnstorfer et al., 2014). In DraNramp, the metal-binding site is unoccupied and exposed to the intracellular side (see Figure 2A). Thus the structure resembles a substrate-free inward-facing conformation. Accordingly, DraNramp and ScaNramp superimpose well with a root-mean-square distance of 1.54 Å over 279 Ca atoms. Clade A members such as DraNramp have a four-residue deletion near the TM9 N terminus in comparison with clade C members such as ScaNramp, resulting in a shorter helix (Figure S1D) (Cellier, 2012). The main difference between the two structures is the position of scaffold helix TM5 (Figure 1C), more angled relative to the membrane plane in DraNramp, perhaps influenced by the presence of TM1a in the structure.

As in other LeuT-fold proteins (Figure 1E), DraNramp TM1a is bent outward and lies nearly parallel to the membrane plane, providing substrate-binding residues as observed in ScaNramp (Ehrnstorfer et al., 2014). In DraNramp, the metal-binding site is unoccupied and exposed to the intracellular side (see Figure 2A). Thus the structure resembles a substrate-free inward-facing conformation. Accordingly, DraNramp and ScaNramp superimpose well with a root-mean-square distance of 1.54 Å over 279 Ca atoms. Clade A members such as DraNramp have a four-residue deletion near the TM9 N terminus in comparison with clade C members such as ScaNramp, resulting in a shorter helix (Figure S1D) (Cellier, 2012). The main difference between the two structures is the position of scaffold helix TM5 (Figure 1C), more angled relative to the membrane plane in DraNramp, perhaps influenced by the presence of TM1a in the structure.
Cysteine Accessibility Scanning Reveals an Outward-Facing Metal-Permeation Pathway

To identify a metal-permeation pathway, we created a panel of sequential single-cysteine mutants spanning DraNramp TM1, 3, and 6, which typically line the inward- and/or outward-facing permeation pathway in LeuT-fold transporters (Shi, 2013). The mutations were introduced in the C382S background, which removed the lone endogenous cysteine while retaining full activity (Figure S2E). We measured in vivo accessibility to thiol-specific modifier N-ethylmaleimide (NEM) or inner membrane-impermeable modifier 2-(trimethylammonium)ethyl methane thiosulfonate bromide (MTSET) (Figures S2A and S2B), which both specifically react with aqueous-exposed cysteines (Kaback et al., 2007; Karlin and Akabas, 1998). We classified the cysteine positions in three groups: (1) NEM- and MTSET-reactive residues were deemed extracellularly exposed in at least one DraNramp conformation; (2) NEM-reactive but MTSET-protected residues as intracellularly accessible; and (3) NEM-protected as buried (Figures S2C and S2D and Table S1). We observed high NEM accessibility all along TM1a and 6b, consistent with the large aqueous vestibule below the metal-binding residues in our structure. Between the metal-binding site and the extracellular face, we observed a helical pattern of accessible positions on TM1b, 3, and 6a that line the bundle-scaffold interface (Figure S2D). As many of these NEM-reactive positions are buried in the inward-facing structure (Figure 2A), we conclude that they face an aqueous pathway for periplasmic metal ions to reach the binding site in an alternate outward-open conformation. Thus, our cysteine accessibility measurements, rather than describing a single state, instead provide a composite picture of multiple conformations that DraNramp cycles through in the native membrane.

A Crystallization Construct Mutation Locks the Transporter in an Inward-Open State

We exploited this knowledge of the extracellular metal-permeation pathway to select A61C as a reporter to assess the conformational preferences of various DraNramp mutants (Figures 2A and 2B). A61C was accessible to inner membrane-impermeable modifier MTSET (Figure S2B), indicating that it faces a periplasmic aqueous environment, and A61C/C382S showed WT-level Co2+ transport (Figure S2E). In contrast, our crystallization construct, which contained entropy-reducing mutation patches in three intracellular loops: (1) QK169-70HH, (2) EEK251-3YYY, and (3) RR398-9HH, as well as an N-terminal 25-residue deletion (Figure S2F), did not transport Co2+ (Figure 2C). Patch 2 on its own completely eliminated Co2+ uptake (Figure 2C). This mutation is in intracellular loop 6–7, far from the metal-binding site; thus we tested A61C accessibility over a range of NEM concentrations to probe for conformational preference. Unlike the concentration-dependent increase in NEM modification of A61C for WT DraNramp, A61C was essentially not modified in the patch 2 background (Figures 2D and S2G). Therefore, the patch 2 mutant protein cannot switch to the outward-open state, which explains its loss-of-function phenotype, as substrate ions cannot reach the binding site (below A61C) from the outside. The patch 3 mutant had WT-level Co2+ transport and A61C modification, while the patch 1 mutant had

Figure 2. Cysteine Accessibility Scanning Reveals the Outward Metal-Permeation Pathway that Is Sealed Shut in the Crystallized Construct

(A) Internal slice of the inward-facing DraNramp structure, including solvent accessibility of a panel of cysteine mutants spanning TM1, 3, and 6 using NEM. Spheres show Cx positions of highly NEM-protected (gray), outward-accessible (also MTSET and MTSEA-modified; red), inward-accessible (also MTSEA- but not MTSET-modified; cyan), or only NEM-accessible (black) residues. Accessibility is assessed as >50% NEM modification in at least two separate experiments. Many outward-accessible residues, including A61C, are buried in our inward-open structure, suggesting they line an aqueous passage to the metal-binding site (approximate location labeled *) in an alternate outward-open conformation. (B) The proposed conformational equilibrium of DraNramp, in which A61C is solvent-accessible in the outward-open state, but buried (and thus NEM-protected) in the inward-open conformation. (C) The patch mutants in the crystallized DraNramp construct, tested alone or in combinations, have varying effects on in vivo Co2+ transport. While the 25-residue N-terminal truncation and patch 3 (RR398-9HH) did not impair function, patch 1 (QK169-70HH) reduced transport and patch 2 (EEK251-3YYY) completely eliminated transport.
Figure 3. Glycine-to-Arginine Mutations Impair Transition Metal Transport in Both Human Nramp2 and DraNramp

(A and B) Sequence logos of the TM1a region (A) showing that G45 (shaded green with *), G75 in human Nramp2 on TM1a is absolutely conserved in Nramps, and a TM4 segment (B) showing that G153 (shaded purple with *), G185 in human/mouse/rat Nramp2 is generally a small amino acid. Logos were generated from an HMMER alignment of 2,691 sequences using DraNramp to search the UniProt database, with an E value cutoff of 1 x 10⁻8. Dra. D. radiodurans MnhH; Sca. S. capitis MnhH; Hs2. Homo sapiens Nramp2; Mm2. Mus musculus Nramp2; and Rn2. Rattus norvegicus Nramp2.

(C and D) Fura-2 fluorescence quenching traces showing severe loss of function (no transport activity above baseline) for both G-to-R mutants compared with WT human Nramp2 for transport of the transition metals Fe²⁺ (C) and Cd²⁺ (D) in transfected HEK cells. Traces are representative of at least three independent transfection experiments.

(E) Relative Fe²⁺ uptake of E. coli expressing the analogous G-to-R DraNramp mutants also showed significantly decreased transport activity compared with WT. Plotted are averages ± SD (n = 3).

(F) Both G-to-R DraNramp mutants had decreased Cd²⁺ transport when reconstituted into proteoliposomes. Traces are representative of three experiments. See also Figure S3.

both slightly reduced Co²⁺ transport and A61C accessibility (Figures 2C and 2D). This clear correlation of impaired metal uptake with loss of A61C accessibility further implicates the opening of the interface between the bundle and scaffold as an essential conformational change within the Nramp transport cycle.

Anemia-Causing Mutations Impair Metal Transport in Human Nramp2 and DraNramp

Naturally occurring glycine-to-arginine mutations G75R in TM1a in human Nramp2 and G185R in TM4 in mouse and rat Nramp2 all cause anemia (Blanco et al., 2009; Fleming et al., 1997, 1998). The TM1a glycine is absolutely conserved; the TM4 glycine is generally conserved as a small residue (Figures 3A and 3B). When introduced in human Nramp2, both G-to-R mutations abrogated transport of the physiological substrate Fe²⁺ (Figure 3C), as well as Cd²⁺, Co²⁺, and Mn²⁺ (Figures 3D, S3A, and S3B), as detected in transfected HEK cells using the metal-binding fluorescent dye Fura-2. Similarly, the analogous disease-mutant mimics in DraNramp significantly impaired Fe²⁺ transport as detected colorimetrically in DraNramp-expressing Escherichia coli (Figure 3E), and reduced Cd²⁺ (Figure 3F) and Mn²⁺ transport (Figure S3C) as detected with Fura-2 using purified DraNramp reconstituted into proteoliposomes. In both homologs, the TM1a G-to-R mutant expressed similarly to WT, while TM4 G-to-R mutant expression was reduced (Figures S3D and S3E), which could contribute to the loss-of-function phenotype in the in vivo assays. However, the DraNramp G153R mutation clearly impaired both Cd²⁺ and Mn²⁺ transport in proteoliposomes with normalized protein concentrations (Figure S3F). Notably, the G185R mutation in mouse Nramp2 similarly impaired Fe²⁺ transport (Su et al., 1998). These consistent loss-of-function data for the analogous mutants indicate that DraNramp is a useful model to further investigate how these disease-causing mutations inhibit transport.

The G153R Mutation Alters the Conformational Equilibrium and Metal Selectivity

G153 is far (~20 Å) from the metal-binding site, near the top of TM4 within the scaffold (Figure 4A). We tested the in vivo Co²⁺
uptake activity of various substitutions at this position. G153R, and the similarly large and positively charged G153K, were most impaired (Figure 4B). In contrast, replacing G153 with large aromatics (G153F and G153W) did not significantly alter Co\textsuperscript{2+} uptake (although Fe\textsuperscript{2+} uptake by G153F was somewhat reduced; Figure S4B). A negatively charged glutamate (G153E) reduced uptake much more than a polar glutamine (G153Q), although less than G153R. Overall, charged side chains caused the largest reductions in transport activity.

In our inward-open structure, modeling a bulky substitution at position 153 leads to major steric clashes with nearby residues such as I142 on TM3, indicating that G153R or similarly large substitutions likely alters DraNramp conformation at least locally. We therefore compared outward-open reporter A61C accessibility for this G153X panel (Figures 4C and S4C). All mutations resulted in increased NEM modification of A61C, with even more pronounced effects for G153F and G153W than for G153R. These data alone cannot distinguish between two scenarios: (1) increased occupancy of the outward-open state with concomitant decreased inward-open state occupancy; or (2) local structural changes that increase A61C accessibility in a protein that can still undergo conformational cycling. We further investigated this effect in Figure 6 (below).

Previous work showed that in mouse Nramp2 the G185R mutation decreased Fe\textsuperscript{2+} transport, but surprisingly enabled Ca\textsuperscript{2+} transport (Xu et al., 2004). This could occur through either distortion of the conserved metal-binding site or opening of an alternative metal-permeation pathway. We first tested whether the residual Fe\textsuperscript{2+} transport by G153R DraNramp relied on the conserved metal-binding site, introducing an alanine substitution for each binding-site side chain into the WT or G153R background. Either a D56A or N59A substitution abolished Fe\textsuperscript{2+} transport in both backgrounds, while M230A had negligible effects in both cases (Figure 4D), consistent with our previous results (Bozzi et al., 2016). Thus, G153R still relies on the conserved metal-binding residues for Fe\textsuperscript{2+} transport. We next tested whether
G153R perturbed the selectivity of this binding site by measuring Fe\(^{2+}\) transport in the presence of competing Ca\(^{2+}\) for WT, G153R, M230A, and G153R/M230A (Figure 4E), as well as G153F (Figure S4D). As expected, M230A, lacking the methionine “selectivity filter” that excludes alkaline earth metals (Bozzi et al., 2016), was more susceptible to Ca\(^{2+}\) competition than WT. Interestingly, G153R was even more susceptible to Ca\(^{2+}\), and the G153R/M230A double mutant was most susceptible, as 4 mM Ca\(^{2+}\) effectively abolished Fe\(^{2+}\) uptake. This suggests that G153R indeed perturbs the metal-binding site in favor of Ca\(^{2+}\) over Fe\(^{2+}\) (Figure 5C) nearly as much as G45R. Furthermore, single-cysteine reporter A61C was essentially fully protected in G45F, indicating that the outward-open state was rarely if ever sampled, while A61C accessibility in G45R suggested a shifted conformational preference, strongly favoring the inward-open state, thus explaining the severe metal-transport impairment of G45R (Figure 3). Consistently, the bulky uncharged G45F substitution impaired in vivo uptake of Co\(^{2+}\) (Figure 5B) and Fe\(^{2+}\) (Figure 5C) nearly as much as G45R. Furthermore, single-cysteine reporter A61C was essentially fully protected in G45R, indicating that the outward-open state was rarely if ever sampled, while A61C accessibility in G45F suggested a shifted conformational preference, strongly favoring the inward-open state relative to WT (Figure 5D). Thus, decreased A61C accessibility of G45 substitutions correlated with decreased metal transport. This is similar to the patch mutant phenotypes (Figures 2C and 2D), especially patch 2, which just like G45 is located in the intracellular half of the bundle, indicating the importance of proper conformational cycling for protein function.

Mutations at Glycine Positions Illustrate the Conformational Change Process in DraNramp

To further explore the DraNramp conformational cycle, we investigated how G45R and G153F altered solvent accessibility for a panel of inward- or outward-accessible single-cysteine substitutions that retained significant transport activity (Figures S5A and S5B). Accessibility of outward reporters A61C and L140C was correlated; G45R greatly decreased while G153F greatly increased Ca\(^{2+}\) transport (Figure S4E) but increased Ca\(^{2+}\) transport compared with WT (Figure 4F). Of note, the other disease-mimic G45R, impaired both Ca\(^{2+}\) and Cd\(^{2+}\) transport (Figure 4F), thus the metal specificity perturbation is unique to G153R. In summary, the G153R mutation in our bacterial DraNramp model system alters the conserved metal-binding site in a manner that both improves Ca\(^{2+}\) transport and impair transition metal transport, analogously to mouse Nramp2.
increased accessibility (Figure 6A). This suggests that G153F favors a conformational state in which part of the bundle-scaffold interface above the metal-binding site is highly exposed (perhaps by dislodging loop 7–8 that blocks solvent access to A61 and L140 in the inward-open structure). Closer to the metal-binding site within the bundle-scaffold interface, outward-accessible G223C and A133C were also fully protected in G45R, further supporting our inward-locked model for G45R (Figure 6A). However, at those cysteine positions G153F only had a modest effect, slightly increasing accessibility. Finally, neither G45R nor G153F altered accessibility of R211C on extracellular loop 5–6.

Accessibility was also unaltered at T130C, on TM3 adjacent to the unwound regions of TM1 and 6, indicating that the metal-binding site remains solvent-accessible in both G153F and G45R (Figure 6A). To probe for water-excluding structural changes in the cytoplasmic vestibule, we compared NEM accessibility of five cysteines located below the metal-binding site in the WT, G45R, and G153F backgrounds (Figure 6B). Accessibility was the same in all backgrounds for S51C on TM1a, A267C on TM7, and A334C on TM8. For I52C and A53C on TM1a just below the metal-binding D56, accessibility was higher in G45R, suggesting that these positions form part of the cytoplasmic gate regulating access to the metal-binding site. We observed only minor perturbations at all inward-reporter positions for G153F. Thus, this mutation likely does not lock the protein in the outward-facing state with a closed intracellular gate, consistent with its high metal-transport activity (Figures 4B and S4B). In summary, the intracellular gate likely includes the TM1a C terminus, although a more complete picture awaits a truly outward-locked mutant. On the extracellular side, our crystal structure

---

Figure 6. Mutations of Conserved Glycines Shift the Conformational Landscape of DraNramp

(A) Cysteine modification as a function of NEM concentration for five extracellularly accessible reporters, mapped on a top view of the structure (left). A61C data are repeated from Figures 3C and 4D for comparison.

(B) NEM modification for five intracellularly accessible cysteine reporters, as well as T130C, which could not clearly be assigned as intracellularly or extracellularly accessible. Reporter positions are indicated on the structure, viewed down the cytoplasmic vestibule.

* indicates the metal-binding site. All data are averages ± SD (n ≥ 4). See also Figure S5.
and cysteine accessibility results clearly demonstrate a ~15 Å thick, solvent-excluding gate that remains firmly closed in the G45R mutant.

**TM1a Movement Is Essential to the DraNramp Transport Cycle**

To determine the functional importance of the DraNramp N terminus, we designed a truncation series that eliminated 25–49 residues (Figures S6A–S6C). Co\(^{2+}\) uptake activity was at WT levels up through the \(\Delta N34\) construct, and slight reductions in transport accompanied each further truncation for \(\Delta N37, \Delta N40,\) and \(\Delta N43\) (Figure S6). In contrast, \(\Delta N46\) and \(\Delta N49\), which both truncate beyond the TM1a N terminus at the invariant G45, showed pronounced drops in Co\(^{2+}\) uptake activity.

To further assess the importance of TM1a movement for metal transport, we compared Co\(^{2+}\) uptake for single-cysteine mutants, either unmodified or pre-modified with NEM (Figure 7A). For cysteine-less mutant C382S, WT (with the inaccessible C382), and extracellular loop control R211C, NEM treatment did not impair transport. However, for six of seven TM1a positions where the cysteine mutant retained transport activity, NEM modification greatly impaired transport. Thus, while those cysteines were highly accessible in at least one conformation, they must move into a more congested environment as part of the cation-transport cycle, which NEM modification sterically blocks. This phenomenon was not observed for positions preceding TM1a (residues 40–44, disordered in the structure). Cysteine substitution alone impaired transport at four positions: G45C, consistent with the importance of this conserved glycine and loss of function of G45R and G45F (Figures 3 and 5); D56C and G58C, which disrupt the metal-binding site; and A50C, a surprising result given the subtle size increase, which may reflect how close this face of TM1a must approach TM7 and/or the scaffold (TM8) as the inside gate closes in the outward-open state. Surprisingly, we did not observe NEM-dependent impairment for the functional cation transport in the TM1b positions A61C and E65C, the first of which clearly lines the outward metal-permeation pathway and is buried in the inward-open state. An A61W mutant similarly retained WT-level Co\(^{2+}\) transport, showing that DraNramp can indeed tolerate added bulk at this position (Figure S6D). However, pre-modifying A61C with the positively charged MTSET selectively inhibited Co\(^{2+}\) uptake (Figure S6G), thus some perturbation of the outward-metal-permeation pathway at position A61 can indeed disrupt transport.

Finally, to further test the functional importance of TM1a movement, we substituted tryptophan (an alternative way of adding steric bulk) at each of the six TM1a positions where NEM modification of cysteine mutants drastically impaired transport. Analogously to G45R, these tryptophan mutations greatly impaired Co\(^{2+}\) transport and fully protected the A61C outward-open reporter (Figures S6D–S6F), indicating that bulky modifications on TM1a lock the protein in an inward-facing conformation. In summary, these results indicate that TM1a movement is an integral part of Nramp conformational rearrangement and is thus required for metal transport. From all of our results, we propose a model of Nramp conformational change in which significant inward movement of TM1a toward the scaffold is crucial to opening the outside interface between the bundle (TM1b and 6a) and scaffold (TM3, 8, and 10) to allow metal ions (and bulk water) to access the binding site from the outside (Figure 7B).

**DISCUSSION**

Using X-ray crystallography, cysteine accessibility, and metal-transport measurements, we developed DraNramp as a model to understand the conformational change process for the Nramp family of divalent metal transporters. The DraNramp structure, while also a LeuT fold in an inward-facing conformation, complements the ScaNramp structure (Ehrnstorfer et al., 2014) by identifying the location of the functionally important TM1a and (with cysteine-scanning mutagenesis) the external metal-permeation pathway. It also provides a structural example of a second distinct evolutionary clade within the Nramp family. Furthermore, our structure showed the locations of two highly conserved glycines where mutations to arginine cause anemia in either rodents (Fleming et al., 1997, 1998) or humans (Blanco et al., 2009) and impair transport in both human Nramp2 and DraNramp (Figure 3).

The G153R mutation on DraNramp TM4 mimics the phenotype first observed in mouse Nramp2 (Xu et al., 2004), altering metal-transport selectivity, despite these two homologs having only 29% sequence identity. G153R perturbs the conserved metal-binding site, thus reducing transport of the typical transition metal substrates of Nramp and increasing Ca\(^{2+}\) transport (Figure 4). A bulky residue cannot be accommodated at position 153 in our inward-facing structure. Our data are consistent with the G153R mutation disturbing the scaffold in a manner that interferes with closing of the extracellular metal-permeation pathway (Figure 6). Precisely how a mutation 20 Å from the binding site increases Ca\(^{2+}\) permeability remains undetermined, but interestingly G153R and M230A, which remove the sulfur ligand responsible for excluding alkaline earth metals (Bozzi et al., 2016), have additive effects (Figures 4 and S4). Other residues besides the four previously identified may be important to metal binding and/or selectivity and, in at least one other Nramp homolog, mutations distant from the metal-binding site affect substrate preference (Pottier et al., 2015).

G45R on TM1a forms a steric wedge that prevents the protein from reaching the outward-open state (Figure 5). Moreover, our results imply that the unfettered movement of TM1a is essential to the conformational rearrangement that must occur to allow metal transport (Figures 7 and S6).

Our study adds to a wealth of structural and functional information available for LeuT-fold secondary transporters (Penmatsa and Gouaux, 2014; Shi, 2013) that expanded on the original “rocking bundle” model of alternating access in which the bundle (TM1, 2, 6, and 7) moves relative to the scaffold (Forrest and Rudnick, 2009; Forrest et al., 2008). Comparison of outward- and inward-open LeuT structures (Krishnamurthy and Gouaux, 2012) showed TM1a swinging up by 45° and a smaller TM6b motion exposing the substrate-binding site to the cytosol. However, the physiological relevance of this large TM1a movement in LeuT is disputed: while some movement is essential to substrate release (Zhao et al., 2011), the Y268A mutation on TM6b in the crystallized construct disrupts a hydrogen-bonding network in the inward-facing state and stabilizes a conformation not highly sampled by the WT protein (Kazmier et al., 2014b).
Furthermore, these LeuT structures and functional studies (Claxin et al., 2010) are in agreement with our DraNramp cysteine accessibility data. The structures showed that movement of TM1b and 6a away from the scaffold opens an aqueous pathway to the binding site, consistent with molecular dynamics simulations also supporting a less drastic tilt in a lipid bilayer environment (Grouleff et al., 2015). In DraNramp, our inward-locking patch 2 mutant and/or crystal-packing interactions with the Fab could analogously stabilize the observed profound TM1a kink. Additional Nramp structures will help to clarify this issue.

We nevertheless predict that TM1a undergoes significant displacement during the conformational change process, given its functional importance in DraNramp. In addition, the LeuT structures showed that movement of TM1b and 6a away from the scaffold opens an aqueous pathway to the binding site, in agreement with our DraNramp cysteine accessibility data. Furthermore, these LeuT structures and functional studies (Claxton et al., 2010) illustrated the contribution of loop 7–8 to closing the metal-binding site and lead to different environmental controls of its conformational landscape, perhaps with a protonation event substituting for the Na+ coordination that governs LeuT conformational preference (Malinauskaite et al., 2014; Tavoulari et al., 2016). Ultimately, a complementary outward-open Nramp structure will help answer outstanding questions regarding the Nramp transport cycle.

Figure 7. TM1a Movement Is Essential to the Conformational Change that Opens the Outward Metal-Permeation Pathway, thus Enabling Nramp Metal Transport

(A) Initial (6 min) in vivo Co²⁺ transport (minus EV control) for accessible single-cysteine mutants along TM1 that were either left unmodified (black bars) or pre-reacted with NEM (3 mM; green bars). NEM modification greatly impaired transport for six of the seven TM1a positions where cysteine mutants had high transport activity. Data are averages ± SD (n = 3). Western blots show that all introduced cysteines were efficiently NEM labeled, as preincubation with NEM (+) prevented formation of 5 kDa PEG maleimide DraNramp (upper band in the [-] lanes). R211C on extracellular loop 5–6 was readily NEM modified without affecting activity. Endogenous C382 on TM10 in WT was not modified by NEM, and thus was fully modified by 5 kDa PEG maleimide. The cysteine-less C382S is not labeled by either NEM or 5 kDa PEG maleimide. (B) Model of the conformational change process in DraNramp. Our metal transport and cysteine accessibility results demonstrated that the unencumbered TM1a movement is essential for conformational change into the outward-facing state, including evicting loop 7–8 which caps the outside metal-permeation pathway and opening the interface between the bundle (TM1b and 6a) and scaffold (TM3, 8, and 10), thus allowing periplasmic metal ions to reach the binding site in the unwound regions of TM1 and 6 at the center of the membrane plane. See also Figure S6.
EXPERIMENTAL PROCEDURES

Cloning of Nramp Constructs

The DraNrramp sequence was inserted into the Ndel and Noti sites of pET21-N18H and human Nrramp2 into pCDNA3 vectors as described previously (Bozzi et al., 2016). Mutagenesis was performed using QuickChange protocols (Stratagene), and confirmed by sequencing.

DraNrramp Protein Purification

DraNrramp C41(DE3)-cells were grown at 37°C in 12 L of terrific broth with 10% (w/v) glycerol and 100 mg/L ampicillin, inoculating with 1:50 overnight culture to an optical density at 600 nm of 1.0, induced with 100 μM isopropyl-β-D-thiogalactoside for 4 hr. Proteins were purified at 4°C. Protein samples were mixed with 1 ml sonication buffer and frozen in liquid nitrogen. Debris was removed by 35 min centrifugation at 140,000 g and concentrated using a 50 kDa cutoff concentrator. DraNrramp-Fab crystals were grown by vapor diffusion against 0.1 M sodium acetate (pH 4.5), 0.05 M magnesium acetate, 24% PEG 400, and 0.4%–1% β-octylglucoside in 1:1 volume ratio of sitting drops at 4°C. Crystals derivatized with 1 mM OsmCl2 for 1 day turned dark brown and were frozen in liquid nitrogen.

Diffraction data were collected from three crystals at the Advanced Photon Source ID24-E and C beamlines, using vector scanning and full transmission, processed with HKL2000 (Otwinski and Minor, 1997), and merged to improve completeness. For initial phasing, a ScAnramp-based homology DraNrramp model built using SWISS MODEL (Biasini et al., 2014) and the Fab structure were used as MR search models in Phaser (McCoy et al., 2007). Experimental phase information from single-wavelength anomalous diffraction from the osmium-soaked crystals was determined using AutoSol in PHENIX (Adams et al., 2010). The resulting osmum sites were combined with the MR solution and data containing the experimental phasing information were used for refinement. Coot (Emsley and Cowtan, 2004) was used for model building. Initial rounds of refinement used xMDFF, with input files prepared in VMD (Humphrey et al., 1996) using the MDFU-GUI (McCgreery et al., 2016) with default molecular dynamics parameters (McCgreery et al., 2014), improving Rwork/Rfree from 0.50/0.54 to 0.41/0.45. The model was further refined using PHENIX with additional local refinements using xMDFF. TM1a was placed using a combination of xMDFF and steered molecular dynamics (SMD) simulations. As with the CiVSP voltage sensor protein (Li et al., 2014), a helix-screw motion was induced in SMD simulations using the orientation quaternion (Jiang et al., 2014) of the helix as a collective variable, while simultaneously using electron density restraints. The TM1a helix position featuring the lowest R value was identified, resulting in a 2% increase in the local cross-correlation. It is also consistent with cysteine accessibility data (Figure 6). After final rounds of refinement in PHENIX, the final model includes residues 43–165, 176–236, 310–341, 353–428 for DraNrramp, 1–129 + 132–213 and 1–213 for the Fab heavy and light chains, respectively, and three osmium ions. Figures were made in PyMOL (Schrodinger).

Cysteine Accessibility Measurements

Adapting the cysteine-labeling protocol from Tetsch et al., (2011), E. coli expressing single-cysteine DraNrramp variants were exposed to thiol modifiers in 100 mM Tris (pH 7), 60 mM NaCl, 10 mM KCl, 0.5 mM MgCl2, and 0.75 mM CaCl2. All incubations were at 37°C. To determine overall accessibility, 3 mM NEM was applied for 60 min. To determine inside versus outside accessibility, 3 mM MTSEA or MTSET was applied for 30 min following by 1.5 mM NEM for 30 min. For all NEM-gradient experiments, the indicated NEM concentration was applied for 15 min at room temperature. Excess cysteine was added to quench reactions. Cells were washed twice, resuspended and incubated 1 hr in 100 mM Tris (pH 7), 6 mM urea, 0.5% SDS, and 0.5 mM DTT, incubated with a 2-fold excess of 5 kDa PEG maltamide (Creative PEGWorks), then quenched with sample buffer containing β-mercaptoethanol. Protein was detected in western blots using an Alexa 647-conjugated anti-His antibody (Qiagen) and a Typhoon Imager (GE Healthcare), and background-subtracted band intensities (I) were measured using ImageJ64. The upper (5-kDa PEG maltamide modified) to lower band (NEM modified) ratio was determined and compared with the no-NEM sample ratio (defined as maximal upper-to-lower band ratio = 0% NEM modified) to calculate the NEM-modified cysteine fraction using: modified-cysteine fraction = 1 – [(upper + lower)/upper + lower] × [upper-no-NEM/(lower-no-NEM + upper-no-NEM)].

Metal Uptake Assays

Metal-transport experiments in proteoliposomes, E. coli, and HEK293T cells were performed as described previously (Bozzi et al., 2016). See the Supplemental Information for additional description.

ACCESSION NUMBERS

The accession number for the DraNrramp crystal structure reported in this paper is PDB: 5KTE. The unprocessed diffraction images used to determine the Fab and DraNrramp structures were deposited in the Structural Biology Data
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.str.2016.09.017.

AUTHOR CONTRIBUTIONS

R.G. oversaw and designed the research with W.A.W., L.B.B., and A.T.B.; W.A.W. and E.R.G. generated mAbs, which H.L.P. supervised; W.A.W. obtained DraNramp-Fab crystals and all diffraction data; L.B.B. purified DraNramp mutants and determined the DraNramp-Fab structure; L.B.B. and A.T.B. developed in vitro metal-transport assays; A.T.B. made most Nramp mutants, performed all cysteine accessibility and metal-transport experiments, and analyzed the resulting data; A.S. performed the xMDOFF and SMD simulations, which K.S. supervised; A.T.B., L.B.B., and R.G. wrote the manuscript, with input from all authors.

ACKNOWLEDGMENTS

We thank Alexandra Rojek for selecting and cloning several single-cysteine DraNramp mutants, Brandon Lee for help with developing the NEM pre-modification cobalt uptake assay, Jack Nicoludis, Christina Zimanyi, and members of the Gaudet Lab for discussions. The work was funded in part by a Basel O’Connor Starter Scholar Research Award from the March of Dimes Foundation (to R.G.), grant NIH 5P41GM104601 (to K.S.), and a Beckman Postdoctoral Fellowship (to A.S.). We gladly acknowledge supercomputer time from the Texas Advanced Computing Center via Extreme Science and Engineering Discovery Environment grant NSF-MCA03S028. We thank the NE-CAT beamline staff at the Advanced Photon Source (Argonne, IL, USA) for help with data collection. NE-CAT is funded by NIH (P41 GM103403 and S10 RR029205), and the Advanced Photon Source by the U.S. Department of Energy (DE-AC02-06CH11357).

Received: July 13, 2016
Revised: September 27, 2016
Accepted: October 7, 2016
Published: November 10, 2016

REFERENCES


