**High-resolution structures with bound Mn2+ and Cd2+ map the metal import pathway in an Nramp transporter**

**Shamayeeta Ray1, Samuel P. Berry1, Eric A. Wilson2, Casey H. Zhang1,3, Mrinal Shekhar4, Abhishek Singharoy2, and Rachelle Gaudet1\***

**Affiliations**

1Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA USA

2School of Molecular Sciences, Arizona State University, Tempe, AZ USA

3Present address: Grossman School of Medicine, New York University, New York, NY USA

4Broad Institute of MIT and Harvard, Cambridge, MA USA

\* To whom correspondence should be addressed (gaudet@mcb.harvard.edu)

**This file contains Supplementary Tables 1-9**

**Supplementary file 1a.** **Construct, precipitant, and soaking solutions used for each structure**

|  |  |  |
| --- | --- | --- |
| Structure (Construct•substrate)a | Precipitant solution | Soaking solutionc |
| ΔN31-WT (no metal) | 28% PEG 400  0.1 M MES (pH 6.7)  50 mM succinic acid (pH 6.0)  5 mM spermidine (pH 7.0) |  |
| ΔN31-WTsoak | 28% PEG 400  0.1 M MES (pH 6.3)  50 mM succinic acid (pH 6.0)  5 mM spermidine (pH 7.0) | 28% PEG 400  0.1 M MES (pH 6.5)  50 mM succinic acid (pH 6.0)  20 mM spermidine (pH 7.0) |
| ΔN31­-WT•Mn2+ | 24% PEG 400  0.1 M MES (pH 5.9)  50 mM succinic acid (pH 6.0)  20 mM spermidine (pH 7.0) | 28% PEG 400  0.1 M MES (pH 6.5)  50 mM succinic acid (pH 6.0)  20 mM spermidine (pH 7.0)  2 mM MnCl2 |
| ΔN31-WT•Cd2+ | 24% PEG 400  0.1 M MES (pH 6.1)  50 mM succinic acid (pH 6.0)  20 mM spermidine (pH 7.0) | 28% PEG 400  0.1 M MES (pH 6.5)  50 mM succinic acid (pH 6.0)  20 mM spermidine (pH 7.0)  2 mM CdCl2 |
| A47W•Mn2+ (b) | 20% PEG MME 550  0.1 M HEPES (pH 7.2)  0.25 M NaCl  10 mM MnCl2 |  |
| ΔN31-M230A•Mn2+ (b) | 32% PEG 400  0.1 M MES (pH 6.5)  50 mM succinic acid (pH 6.0)  20 mM spermidine (pH 7.0)  10 mM MnCl2 |  |
| ΔN31-D296A•Mn2+ (b) | 28% PEG 400  0.1 M HEPES (pH 6.8)  0.1 M NaCl  10 mM MnCl2 |  |

a ΔN31 refers to a deletion of the N-terminal 31 residues. For simplicity, the “ΔN31” annotation is listed here, but omitted elsewhere in the text.

b Proteins were premixed with 5 mM MnCl2 prior to setting up crystallization trials in precipitants spiked with 10 mM MnCl2.

c Crystals of ‘ΔN31-WT (no metal)’ were soaked in the listed soaking solution overnight prior to harvesting.

**Supplementary file 1b.** **Data collection and refinement statistics for the four supporting new DraNramp structures**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Structure | WT | A47W•Mn2+ | D296A•Mn2+ | G223W•Mn2+ (a) |
| Conformation | Occluded | Occluded | Inward open | Outward open |
| Bound substrate | none | Mn2+ | Mn2+ | Mn2+ |
| PDB ID | 8E5S | 8E6H | 8E6L | 8E6N |
| **Data Collection** |  |  |  |  |
| Beamline | NECAT 24IDC | NECAT 24IDC | GMCA 23IDB | NECAT 24IDC |
| Wavelength (Å) | 0.984 | 0.984 | 1.033 | 0.979 |
| Resolution range (Å) | 41.06-2.38 (2.46-2.38) | 45.35-2.39 (2.47-2.39) | 45.32-3.12 (3.23-3.12) | 39.19-2.40 (2.49-2.40) |
| Space group | P 2 21 21 | P 2 21 21 | P 2 21 21 | C1 21 |
| Unit cell (*a, b, c*) | 58.66, 70.85, 98.34 | 58.98, 70.93, 98.57 | 58.51, 71.64, 98.89 | 105.76, 80.39, 51.75 |
| Unit cell (α, β, γ) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 94.72, 90 |
| Number of crystals | 1 | 1 | 3 | ~15 |
| Total reflections | 74541 (7226) | 61843 (6302) | 27350 (2837) | 49321 |
| Unique reflections | 15762 (1607) | 16817 (1653) | 7004 (687) | 13962 (621) |
| Redundancy | 4.7 (4.5) | 3.7 (3.8) | 3.9 (4.1) | 3.5 (1.7) |
| Completeness (%) | 91.66 (93.38) | 98.57 (99.52) | 89.46 (90.75) | 82.1\* (36.4) |
| Mean *I/σ (I)* | 9.83 (1.60) | 7.92 (1.03) | 6.71 (1.49) | 6.2 (1.6) |
| Wilson *B*-factor | 36.73 | 40.33 | 63.48 | 58.94 |
| *R*merge | 0.192 (1.057) | 0.163 (1.371) | 0.342 (1.216) | 0.186 |
| *R*meas | 0.218 (1.189) | 0.191 (1.588) | 0.386 (1.380) | 0.21 |
| *R*pim | 0.099 (0.528) | 0.097 (0.789) | 0.173 (0.634) | 0.095 |
| CC1/2 | 0.99 (0.51) | 0.98 (0.41) | 0.80 (0.53) | 0.98 (0.58) |
| **Refinement** |  |  |  |  |
| Resolution range (Å) | 41.06-2.38 (2.46-2.38) | 45.35-2.39 (2.47-2.39) | 45.32-3.12 (3.23-3.12) | 39.19-2.40 (2.49-2.40) |
| No. reflections | 15665 (1579) | 16749 (1653) | 6999 (687) | 13963 (621) |
| No. reflections in *R*free | 1565 (159) | 1673 (165) | 910 (90) | 701 (33) |
| *R*work | 0.206 (0.264) | 0.205 (0.299) | 0.219 (0.287) | 0.223 (0.276) |
| *R*free | 0.248 (0.312) | 0.246 (0.371) | 0.272 (0.352) | 0.271 (0.337) |
| Number of atoms | 3403 | 3483 | 3205 | 3310 |
| Protein | 2922 | 2939 | 2861 | 3012 |
| Ligand | 434 | 469 | 323 | 244 |
| Water | 47 | 75 | 21 | 54 |
| Protein Residues | 392 | 392 | 385 | 398 |
| Ramachandran plot |  |  |  |  |
| Favored (%) | 98.72 | 99.74 | 98.16 | 97.73 |
| Allowed (%) | 1.28 | 0.26 | 1.84 | 2.27 |
| Outliers (%) | 0 | 0 | 0 | 0 |
| Rotamer outliers (%) | 0.34 | 1.00 | 0.69 | 1.29 |
| Clashscore | 7.21 | 6.38 | 7.04 | 6.48 |
| RMS (bonds) | 0.003 | 0.002 | 0.002 | 0.002 |
| RMS (angles) | 0.49 | 0.43 | 0.45 | 0.44 |
| Average *B*-factor | 48.09 | 50.38 | 68.03 | 75.00 |
| Protein | 46.20 | 47.30 | 68.42 | 74.11 |
| Ligand | 61.54 | 70.13 | 65.22 | 88.03 |
| Water | 41.47 | 47.56 | 58.65 | 65.83 |
| No. of TLS groups | 7 | 3 | 8 | 5 |

Values in parentheses are for highest-resolution shell. Data for D296A•Mn2+ merge reflections from multiple crystals. Data for the other structures were obtained from a single crystal.

a G223W•Mn2+ has been re-refined from PDB ID: 6BU5, the previously published data collection statistics (Bozzi, Zimanyi, et al., 2019) are reproduced here for completeness.

**Supplementary file 1c. Cα RMSD in Å for all DraNramp structure pairs (number of aligned residues in parentheses)**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Conformation** |  | **Outward open** | | **Occluded** | | | | | **Inward open** | | |
|  | **Structure** | G223W (a) | G223W•Mn2+ | WTsoak | WT | WT•Mn2+ | A47W•Mn2+ | Patch (a,b) | M230A•Mn2+ | D296A•Mn2+ (b) | WT•Cd2+ |
| **Outward open** | G223W (a) |  | 0.97 (396) | 2.29  (359) | 2.29  (356) | 2.33  (361) | 2.39  (359) | 2.57  (320) | 2.45  (356) | 2.38  (344) | 2.32  (347) |
| G223W•Mn2+ | 0.97  (396) |  | 2.34  (348) | 2.38  (350) | 2.39  (351) | 2.47  (353) | 2.67  (320) | 2.62  (355) | 2.47  (339) | 2.47  (398) |
| **Occluded** | WTsoak | 2.29  (359) | 2.34  (348) |  | 0.38  (391) | 0.20  (392) | 0.43  (388) | 1.54  (328) | 0.56  (386) | 0.61  (366) | 0.77  (372) |
| WT | 2.29  (356) | 2.38  (350) | 0.38  (391) |  | 0.39  (392) | 0.42  (391) | 1.69  (329) | 0.71  (387) | 0.82  (367) | 0.95  (374) |
| WT•Mn2+ | 2.33  (361) | 2.39  (351) | 0.20  (392) | 0.39  (392) |  | 0.47  (389) | 1.53  (328) | 0.59  (387) | 0.65  (367) | 0.79  (373) |
| A47W•Mn2+ | 2.39  (359) | 2.47  (353) | 0.43  (388) | 0.42  (391) | 0.47  (389) |  | 1.59  (326) | 0.59  (383) | 0.61  (363) | 0.60  (368) |
| **Inward open** | Patch (a,b,c) | 2.57  (320) | 2.67  (320) | 1.54  (328) | 1.69  (329) | 1.53  (328) | 1.59  (326) |  | 1.59  (339) | 1.45  (340) | 1.52  (341) |
| M230A•Mn2+ | 2.45  (356) | 2.62  (355) | 0.56  (386) | 0.71  (387) | 0.59  (387) | 0.59  (383) | 1.59  (339) |  | 0.38  (372) | 0.48  (376) |
| D296A•Mn2+ (b,c) | 2.38  (344) | 2.47  (339) | 0.61  (366) | 0.82  (367) | 0.65  (367) | 0.61  (363) | 1.45  (340) | 0.38  (372) |  | 0.47  (384) |
| WT•Cd2+ (c) | 2.32  (347) | 2.47  (398) | 0.77  (372) | 0.95  (374) | 0.79  (373) | 0.60  (368) | 1.52  (341) | 0.48  (376) | 0.47  (384) |  |
| a Previously published structures (G223W is PDB ID: 6D91; Patch is PDB ID: 6D9W and so named because it has a patch of mutations in intracellular loops)  b Lower-resolution structures.  c Structures with unmodeled loops. | | | | | | | | | | | |

**Supplementary file 1d. Data collection for anomalous maps**

|  |  |  |  |
| --- | --- | --- | --- |
|  | A47W•Mn2+ | D296A•Mn2+ | WT•Cd2+ |
|  | Occluded | Inward open | Inward open |
| Beamline | NECAT 24IDC | GMCA 23IDB | NECAT 24IDC |
| Wavelength (Å) | 0.984 | 1.033 | 1.904 |
| Resolution range (Å) | 45.35-2.38 (2.46-2.38) | 45.32-3.12 (3.23- 3.12) | 40.28-2.82 (2.92-2.82) |
| Space group | P 2 21 21 | P 2 21 21 | P 2 21 21 |
| Unit cell (*a, b, c)* | 58.98, 70.93, 98.57 | 58.51, 71.64, 98.89 | 58.54, 70.72, 98.00 |
| Unit cell (α, β, γ) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| Total reflections | 62633 (6419) | 27350 (2837) | 32558 (3197) |
| Unique reflections | 17027 (1685) | 7000 (688) | 10017 (989) |
| Redundancy | 3.7 (3.8) | 3.9 (4.1) | 3.3 (3.2) |
| Completeness (%) | 98.61 (99.53) | 89.48 (90.89) | 96.78 (97.63) |
| Mean *I/σ (I)* | 7.83 (1.00) | 6.71 (1.49) | 6.96 (1.27) |
| Wilson *B*-factor | 40.44 | 63.59 | 54.2 |
| *R*merge | 0.166 (1.409) | 0.321 (1.216) | 0.165 (1.024) |
| *R*meas | 0.191 (1.633) | 0.398 (1.380) | 0.195 (1.219) |
| *R*pim | 0.099 (0.813) | 0.232 (0.634) | 0.101 (0.643) |
| CC1/2 | 0.98 (0.38) | 0.80 (0.53) | 0.98 (0.35) |
| Anomalous completeness (%) | 89.9 (90.6) | 78.1 (77.9) | 85.8 (84.8) |
| Anomalous multiplicity (%) | 1.8 (2.0) | 1.9 (2.3) | 1.6 (1.8) |

Values in parentheses are for highest-resolution shell. Mn2+ ions were co-crystallized with the protein, whereas Cd2+ ions were soaked after crystallization (Supplementary Table 1).

**Supplementary file 1e. Distances to metal (Å) for coordinating atoms at the orthosteric site**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Outward open** | **Occluded** | | **Inward open** | | |
| **Coordinating atom** | G223W•Mn2+ | WT•Mn2+ | A47W•Mn2+ | M230A•Mn2+ | D296A•Mn2+ e | WT•Cd2+ | |
| D56 Oδ1 | 2.4 | 2.7 | 2.6 | 2.7 | 2.7 |  | |
| N59 Oδ1 | 3.2 | 2.4 | 2.5 | 2.6 | 2.4 | 2.9 | |
| M230 Sδ | 3.0 | 2.8 | 2.8 |  | 2.7 | 3.4 | |
| A227 O (Cα) |  | 2.5 | 2.2 | 2.4 | 2.2 | 2.8 | |
| A53 O (Cα) | 2.4 | 2.2 | 2.1 |  |  |  | |
| Y54 O (Cα) |  |  |  | 3.1 | 3.5 | 3.2 | |
| water O (Q378)a | 2.7 | 2.3 | 2.2 | 2.5 | 2.3 | 2.9 | |
| water O (M230A)b |  |  |  | 2.3 |  |  | |
| water (Y54)c |  |  |  | 2.2 |  | 3.3 | |
| water (N59)d | 2.6 |  |  |  |  |  | |
| a Conserved water coordinating central binding ion (water/metal) and Oε1 of Q378 (except in G223W•Mn2+ where Q378 is far away to be in coordinating distance).  b Water replacing M230 in M230A•Mn2+ structure.  c Water coordinating central metal ion and Cα carbonyl of Y54.  d Water coordinating central metal ion and Oδ1 and Nδ2 of N59 in G223W•Mn2+.  e Missing coordinating bonds due to low resolution of the D296A•Mn2+ structure. | | | | | | | |

**Supplementary file 1f.** **Coordination number and geometry of metal ions in the orthosteric site**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | | **Coordination number** | **Coordinating atoms** | **Geometry** | **RMSangle (°)** |
| **Outward open** | G223W•Mn2+ | 6 | SO5 | Octahedral | 22 |
| **Occluded** | WT•Mn2+ | 6 | SO5 | Octahedral | 25 |
| A47W•Mn2+ | 6 | SO5 | Octahedral | 23 |
| **Inward open** | M230A•Mn2+ | 7 | O7 | Pentagonal bipyramidal | 36 |
| D296A•Mn2+\* | 6 (7) | SO5 | Pentagonal bipyramidal | 36 |
| WT•Cd2+ | 6 | SO5 | Octahedral | 34 |
| \*Missing postulated coordinating bond to water molecule due to the low resolution of the D296A•Mn2+ structure. | | | | | |

**Supplementary file 1g.** **Binding affinity of metals to various DraNramp constructs**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Protein construct** | **Manganese** | | | **Cadmium** | | |
| **No of sites** | **Kd (µM)** | | **No of sites** | **Kd (µM)** | |
| WT | 2 | 190 ± 30 | 1970 ± 520 | 2 | 55 ± 15 | 220 ± 20 |
| A47W | 2 | 125 ± 5 | 2450 ± 650 | 1 | 150 ± 10 | |
| D56A | 2 | 230 ± 80 | 3800 ± 1100 | 2 | 80 ± 20 | 2250 ± 750 |
| M230A | 2 | 215 ± 65 | 4600 ± 1300 | 1 | 160 ± 20 | |
| G223W | 1 | 440 ± 15 | | No binding | | |
| D296A | 1 | 370 ± 30 | | 1 | 120 ± 1 | |
| D369A | 1 | 420 ± 30 | | 1 | 70 ± 10 | |
| A47W-D296A | 1 | 255 ± 45 | | No binding | | |
| A47W-D369A | 1 | 300 ± 95 | | No binding | | |
| D56A-D296A | 1 | 250 ± 80 | | No binding | | |
| D56A-D369A | 1 | 210 ± 70 | | No binding | | |
| M230A-D296A | 1 | 230 ± 90 | | No binding | | |
| M230A-D369A | 1 | 770 ± 20 | | No binding | | |

The number of sites were assigned as described in and Appendix 1. Each Kd value represents the mean of 2-3 independent ITC experiments and the mean ± SEM is reported (see Appendix 1 for the results of all individual experiments).

**Supplementary file 1h. Primers for Mutagenesis (5’ to 3’ sequence)**

|  |  |  |
| --- | --- | --- |
| **Mutation** | **Primer** | **Sequence** |
| A47W | forward | GGCCGTGGGTCATCGCGTCTATCGCCTACATG |
| reverse | TGACCCACGGCCCGAGAAACGGCAGGATGC |
| D296A | forward | GGCGCCCTGACCACCGCCTAC­CAGAC |
| reverse | GGTCAGGGCGCCCGCGTTTTCCACGTTCTT |
| D369A | forward | ATGGCCCCGTCGTCGGTGCTGATCTTGTCG |
| reverse | CGACGGGGCCATGCCCAGCAGAATGACGAT |
| Y54A | forward | CGTCTATCGCCGCCATGGACCCCGGCAAC |
| reverse | CCATGCCGGCGATAGACGCGATGACCGCC |
| Y54F | forward | CGCCTTCATGGACCCCGGCAACTTTGCG |
| reverse | CCATGAAGGCGATAGACGCGATGACCGCC |
| M230A | forward | CGGTCGCCCCACACGTCATCTACCTGCACTCGGC |
| reverse | TGTGGGGCGACCGTCGCCCCGATGATGCCCAC |
| Q89A | forward | GCGATGGTGATTGCGAACCTCAGCGCC |
| reverse | GGCGCTGAGGTTCGCAATCACCATCGC |
| H232A | forward | ATGCCAGCCGTCATCTACCTGCACTCGGCGCTC |
| reverse | GACGGCTGGCATGACCGTCGCCCCGATGAT |
| H237A | forward | TACCTGGCCTCGGCGCTCACGCAGGGACGC |
| reverse | CGAGGCCAGGTAGATGACGTGTGGCATGACCGT |

**Supplementary file 1i.** **Summary of molecular dynamics simulations**

|  |  |  |  |
| --- | --- | --- | --- |
| **Simulation** | **Time (ns)** | **Starting structure** | **Number of atoms** |
| S1a | 1031.95 | Outward-open, G223W•Mn2+ (PDB ID 6BU5) with Mn2+ removed and residue 223 mutated back to native glycine *in silico* | 104,624 |
| S1b | 617.55 |
| S2a | 769.80 | New WT•Mn2+ inward-occluded structure, with Mn2+ removed | 103,074 |
| S2b | 1022.85 |
| S3a | 1176.00 | New WT•Cd2+ inward-open structure, with Cd2+ removed | 103,372 |
| S3b | 808.80 |