Structure of Bor1 supports an elevator transport mechanism for SLC4 anion exchangers

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Contributed by Robert M. Stroud, August 1, 2016 (sent for review July 11, 2016; reviewed by Olga Boudker and Christopher Miller)

Boron is essential for plant growth because of its incorporation into plant cell walls; however, in excess it is toxic to plants. Boron transport and homeostasis in plants is regulated in part by the borate efflux transporter Bor1, a member of the solute carrier (SLC) 4 transporter family with homology to the human bicarbonate transporter Band 3. Here, we present the 4.1-Å resolution crystal structure of Arabidopsis thaliana Bor1. The structure displays a dimeric architecture in which dimerization is mediated by centralized Gate domains. Comparisons with a structure of Band 3 in an outward-open state reveal that the Core domains of Bor1 have rotated inwards to achieve an occluded state. Further structural comparisons with UapA, a xanthine transporter from the nucleobase-ascorbate transporter family, show that the downward pivoting of the Core domains relative to the Gate domains may access an inward-open state. These results suggest that the SLC4, SLC26, and nucleobase-ascorbate transporter families all share an elevator transport mechanism in which alternating access to both sides of the membrane is provided by Core domains that carry substrates across a membrane.

X-ray structure | SLC4 transporter | Bor1 | Band 3 | membrane protein

The defining feature of transporters is the ability to carry specific molecules across a membrane. The solute carrier (SLC) group comprises a diverse array of transporters grouped into at least 52 families based on function and sequence homology (1). The SLC4 family is termed the bicarbonate transporters and is subdivided into sodium-coupled cotransporters and anion exchanger subclasses. The SLC4 anion exchangers transport ions in an electroneutral manner, most commonly transporting bicarbonate in exchange for chloride. In addition to bicarbonate transporters, the SLC4 transporters include borate efflux transporters, originally discovered in plants (2, 3). Boron is an essential plant micronutrient that is taken up from the soil and participates in the formation of esters found in plant cell walls. Specifically, borate diesters cross-link a primary cell wall component, pectic polysaccharide hnamogalacturonan II (RG-II) and, thus, contribute to plant cell wall stability (4, 5). In excess levels, however, boron is toxic to plants. The regulation of boron by transporters is therefore important for plant viability and has implications for worldwide agriculture. Indeed, there are ongoing efforts to engineer plants that are tolerant of either high or low boron levels in soil (6–8). The transport and regulation of boron levels is regulated partly by Bor1, a boron exporter that loads xylem, such that boron is transported from roots to shoots and leaves (3). The precise chemical nature boron takes control substrate translocation for chloride. In addition to bicarbonate transporters, the SLC4 transporters include borate efflux transporters, originally discovered in plants (2, 3). Boron is an essential plant micronutrient that is taken up from the soil and participates in the formation of esters found in plant cell walls. Specifically, borate diesters cross-link a primary cell wall component, pectic polysaccharide hnamogalacturonan II (RG-II) and, thus, contribute to plant cell wall stability (4, 5). In excess levels, however, boron is toxic to plants. The regulation of boron by transporters is therefore important for plant viability and has implications for worldwide agriculture. Indeed, there are ongoing efforts to engineer plants that are tolerant of either high or low boron levels in soil (6–8). The transport and regulation of boron levels is regulated partly by Bor1, a boron exporter that loads xylem, such that boron is transported from roots to shoots and leaves (3). The precise chemical nature boron takes control substrate translocation for chloride. In addition to bicarbonate transporters, the SLC4 transporters include borate efflux transporters, originally discovered in plants (2, 3). Boron is an essential plant micronutrient that is taken up from the soil and participates in the formation of esters found in plant cell walls. Specifically, borate diesters cross-link a primary cell wall component, pectic polysaccharide hnamogalacturonan II (RG-II) and, thus, contribute to plant cell wall stability (4, 5). In excess levels, however, boron is toxic to plants. The regulation of boron by transporters is therefore important for plant viability and has implications for worldwide agriculture. Indeed, there are ongoing efforts to engineer plants that are tolerant of either high or low boron levels in soil (6–8). The transport and regulation of boron levels is regulated partly by Bor1, a boron exporter that loads xylem, such that boron is transported from roots to shoots and leaves (3). The precise chemical nature boron takes control substrate translocation for chloride.

Results

A. thaliana Bor1 (AtBor1) was overexpressed and purified from Saccharomyces cerevisiae. Initially, crystals of the full-length 704-residue AtBor1 could be grown but with diffraction limited to ~7-Å resolution. One impediment to determining a structure of any macromolecular complex is the presence of native unfolded regions. Secondary structure predictors suggested that the C-terminal region of AtBor1 may be unfolded, which led us to make a series of C-terminal truncations and test for their effect on crystal diffraction.

Significance

The solute carrier (SLC) 4 transporters are membrane proteins that control bicarbonate transport in human red blood cells and regulate borate transport in plants and yeasts. Previously, one member of the SLC4 family, human Band 3, had its crystal structure determined, which showed it in an outward-open state. We report here what is, to our knowledge, the second crystal structure of an SLC4 protein, the plant borate transporter Bor1. Critically, the structure is in an occluded state open to neither side of the membrane. Because it is in a new state, we are able to compare our model with other related structures and deduce structural transitions that provide alternating access to both sides of the membrane for Bor1 and related transporters.

Author contributions: B.H.T.-S. and R.M.S. designed research; B.H.T.-S. performed research; B.H.T.-S. analyzed data; and B.H.T.-S. and R.M.S. wrote the paper.

Reviewers: O.B., Weill Medical College of Cornell University; and C.M., Howard Hughes Medical Institute, Brandeis University.

The authors declare no conflict of interest.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code SL25).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1612603113/-/DCSupplemental.
Superior crystals were obtained with a construct, termed AtBor1–645, in which the last 59 residues were removed. AtBor1–645 crystallized in space group P4_2_2_2 and permitted the collection of diffraction data to 4.1-Å resolution. The data are anisotropic and extend to an overall resolution of 4.1 × 4.1 × 5.4 Å (25). Subsequent molecular replacement searches using the Gate and the Core domains of human Band 3 produced a single molecular replacement solution with a monomer in the asymmetric unit, and this solution recapitulated the dimer around a crystallographic twofold axis, further validating the solution. After restrained refinement in Refmac5 (26), the model was refined to an R_work/R_free of 35.9/39.1% with good stereochemistry (Methods, Fig. 1, and Table 1). We were able to build 399 of 645 residues, or 62% of the primary sequence. The defined structure begins at residue 33 and ends at residue 586. It contains the 14 expected transmembrane helices (TMs), with several loops connecting them left out because they could not be reliably fit to electron density. The most significant difference in sequence between AtBor1 and Band 3 is that Bor1 has a nearly 100-residue insertion between TMs 10 and 11, which appears disordered in our structure. The loops we were able to fit to electron density were built as poly-alanine segments. To show our structure is as free from model bias as possible, we calculated a simulated annealing composite omit map, which shows continuous electron density for all 14 TM helices (Fig. 1B). The 2Fo–Fc electron density is observable for some of the bulkiest side chains (Fig. S1).

Each Bor1 monomer recapitulates a fold seen in Band 3 (24), and in the more distantly related nucleobase-ascorbate transporter (NAT) proteins UraA and UapA (27, 28). Additionally, the SLC26 family has been observed to display the same overall fold, as shown in a recent structure of SLC26Dg (29). As in those structures, Bor1 consists of two distinct domains, the Gate and the Core. The Gate comprises six TMs (5–7 and 12–14, residues 152–254 and 489–586), and provides the entire dimerization interface, which buries 731 Å^2 of surface area per monomer. The Core comprises eight TMs (1–4 and 8–11, residues 33–151 and 291–486) and contains the putative substrate-binding site (Fig. 1).

The reported data are merged from two isomorphous crystals. Values in parentheses are for the highest-resolution shell.

Comparisons of large-scale structural rearrangements of Bor1 domains are surprisingly informative about the quaternary motions that Bor1 is likely to undergo in the course of a transport cycle. A superposition of Bor1 and Band 3 reveals that the TMs of the Gate domains are in essentially the same position (Cα rmsd = 1.6 Å), whereas the Core domains of Bor1 are rotated inward toward the dimer symmetry axis (Fig. 2). In particular, the Core TM helices most proximal to the Gate domain—TMs 1, 3, and 8—are
Comparison with Band 3 shows Core domains rotate relative to Gate 3o f5
PNAS Early Edition

Discussion

The alternating access mechanism for transport was proposed by Jardetzky 50 y ago (31). The underlying idea is that a substrate binds a cavity from one side of the membrane, triggers a conformational change, and then exits from a cavity facing the other side of the membrane. This basic idea has stood up remarkably well over time. Transporters can be grouped into three basic mechanisms: rocker switch, rocking bundle, and elevator (32). The rocker switch mechanism operates much as Jardetzky described, with two sides of a transporter moving around an immobile substrate-binding site. In the rocking bundle model, the substrate-binding site remains immobile while one domain of the protein moves around a less labile domain. The difference between an elevator transporter mechanism and a rocking-bundle

rotated inward toward the Gate domain and downward toward intracellular side. The extracellular-facing ends of those three helices each moved inward toward the Gate by ~8 Å. Because of this observed rigid-body movement, Bor1 is not in an outward-open state as Band 3, but rather occupies an occluded state.

It has been previously observed that the distantly related NAT family shares a similar fold with SLC4 transporters despite sharing only ~10% sequence identity. Whereas the Band 3 structure was determined in an outward-open state, NAT transportsers UapA and UraA were determined in inward-open states with substrates bound (27, 28). Superposition of the TMs of the Gate domains of Bor1 and UapA shows a reasonably close alignment, with a Co rmsd of 3.1 Å (Fig. 3A). The positioning of the Core domains with respect to the Gates, however, reveals further conformational differences with Bor1 (Fig. 3B). In Bor1, the Core is rotated “down” toward the cytoplasmic side relative to Band 3, whereas in UapA, the Core is further down toward the cytoplasmic side relative to Bor1. The putative substrate-binding site moves downward toward the intracellular side by ~5 Å. Because of this vertical transition, the substrate-binding site of UapA is solvent-exposed and, thus, in an inward-open state. The superpositions of Bor1 with the outward-open Band 3 and the inward-open UapA thus appear sufficient to explain how the Core domains might move relative to the Gate domains to provide alternating access.

There is no ligand bound in our structure, but as in the case with Band 3 (24), UraA (27), UapA (28), and SLC26Dg (29), the substrate-binding site likely resides where the ends of the shortened TM helices TM3 and TM10 pass each other. Although our model was improved by keeping most side chains present, the resolution precludes commenting specifically on their conformations and contacts. To determine experimentally which residues might be involved in borate transport, we used a genetic complementation assay challenging the growth of S. cerevisiae on plates supplemented with boric acid. Yeast are a boron-tolerant organism, and the expression of S. cerevisiae Bor1 has been shown to enable yeast growth when challenged with boric acid (30). BOR1 was deleted in S. cerevisiae and shown to be complemented by transforming with the wild-type BOR1 (Fig. 4). Consistent with previous studies (7), transforming with AtBor1 fails to complement and rescue growth; this is suspected to be because AtBor1 exports borate at lower concentrations (9), which are too low to cause toxicity to S. cerevisiae. Analysis of the Band 3 structure, in conjunction with a multiple sequence alignment of human Band 3, ScBor1, and AtBor1 enabled identification of residues in Bor1 possibly involved in substrate binding (Fig. S2). Because we could not complement with AtBor1, we mutated the homologous residues in ScBor1: T145, D347, N391, and Q396. Upon mutating these residues to alanine, complementation assays show that the D347A mutant completely abolishes growth (Fig. 4). D347 is homologous to E681 in human Band 3, a residue critical for substrate transport (17, 18, 20). Additionally, the N391A and Q396A mutants reduce growth relative to BOR1. A T145A mutant, however, does not impact growth relative to BOR1. Importantly, wild-type ScBor1 and all mutants could be expressed and purified under identical conditions, and showed no abnormal migration behavior by size-exclusion chromatography (Fig. S3), indicating that the loss of complementation displayed by the mutants is not due to defects in expression, folding, aggregation, or sorting to the plasma membrane. Thus, the data suggest that the ScBor1 Core domain residues D347, N391, and Q396 are important for substrate transport. These results do not preclude the possibility that other residues in either the Gate or Core domains could be involved in substrate binding and transport; rather, the complementation data represent experimental identification of residues in a borate transporter that may be important for transport activity.
transport model can be subtle. Three hallmark signs of a bona fide elevator mechanism are the following: (i) a relatively rigid, immobile scaffolding domain; (ii) a mobile carrier domain that contains all or nearly all substrate binding; and (iii) a vertical displacement of the substrate-binding site (32). The NAT transporter UapA has been proposed to function as an elevator transporter (28). Bor1, and the SLC4 family in general, also appear to meet these requirements. Both Bor1 and Band 3 structures were determined in the absence of substrate. However, UraA and UapA each were determined with the presence of their substrates, uracil and xanthine, respectively. In both cases, contacts with ligand are mediated by residues in the Core domain. Although the Band 3 structure did not have substrate bound, its likely substrate-coordinating residues are suspected through structural comparisons with UraA and UapA and mutagenesis studies (17, 18, 20, 24). They, along with the Bor1 residues identified through genetic assays we describe here, also belong solely to the Core domain. The data collectively suggest that substrates in SLC4, SLC26, and NAT transporters are bound by the Core domain and not the Gate domain.

The identity of the Gate as a mostly rigid scaffolding domain also appears to fulfill the description of an elevator mechanism. The NAT transporter UraA was the first protein of this fold to have its structure determined. It also served as the origin of the naming of the Gate and Core domains, which have since been used to name other SLC4 family transporters. Among other structures to the literature. The SLC26 family also shares a similar fold to UraA, UapA, Bor1, and Band 3. Unlike the other structures, the furamater transporter SLC26Dg crystallized as a monomer (29). However, prior biochemical studies of other members of the SLC26 family suggest the family is ordinarily comprised of dimers (33, 34). Dimerization by the Gate domain thus appears to be conserved among each of the SLC4, SLC26, and NAT transporter families. It is unclear whether each of the two Cores of Bor1 may move around the Gates independently of one another, or whether there is cooperativity between the two. However, transport studies of Band 3 show that one monomer may transport while the other is blocked by an inhibitor, suggesting that Band 3 monomers operate independently (35, 36). Additionally, in the case of the trimeric amino acid transporter and elevator transporter archetypal type GltpH, individual subunits exist independently of each other (37–39).

The combination of Core domain structural rearrangements and substrate binding residues together suggest that the SLC4, SLC26, and NAT families all use a conserved elevator transport mechanism (Fig. S5). In this scheme, the Core domains can move such that they are open to either the extracellular or intracellular sides, whereas the Gate domains remain relatively static. The vertical displacement of the substrate-binding site in the Bor1 occluded state to either the open-outward or open-inward states is approximately 5 Å each, or 10 Å total vertical displacement between the inward- and outward-facing states. This change is not as large as the 18 Å observed in the trimeric elevator transporter GltpH (40), or the 15 Å in the model of transport by VcINDY (41). Rather, a 10-Å change compares more with the 10-Å vertical displacement observed in the sodium/proton dimeric exchanger NapA, which is also proposed to function as an elevator transporter (42). Thus, the available evidence suggests that the SLC4, SLC26, and NAT family transporters all share a conserved elevator transport mechanism.

Methods

Protein Expression and Purification. A 2-μm plasmid S. cerevisiae expression construct based on p423 GAL1 contained nucleotides coding for the A. thaliana borate transporter Bor1 (UniProt ID: Q8VYR7) with a C-terminal deca-histidine tag preceded by a thrombin cleavage site. Transformed S. cerevisiae (strain DSY-5) were grown at 30 °C in CSM-HisO2 media, to a final galactose concentration of 2%. Cells were harvested after 16 h shaking at 30 °C by spinning at 3,500 × g for 15 min. Yeast pellets were resuspended in a buffer containing 50 mM Tris pH 7.0, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride for protease inhibition. Cells were lysed by bead beating with 0.5-mm glass beads for six 1-min pulses separated by 2-min rest periods. The glass beads were filtered from the homogenate and washed with a 2× buffer for a final lysis buffer of 50 mM Tris pH 7.0, 700 mM NaCl, 10% glycerol, 1 mM EDTA, and 1 mM PMSF. The homogenate was centrifuged for 25 min at 18,000 × g, followed by sedimentation of membranes by ultracentrifugation at 185,000 × g for 150 min. Membrane pellets were resuspended in a volume of 15 μL of buffer per gram of membrane, such that 22 μg of DDM per gram of membrane was a concentration of 1.5% DDM by volume. Membranes were solubilized with a stir bar for 60 min at 4 °C. Unsolubilized material was removed by ultracentrifugation for 142,000 × g for 20 min, 50 mM Tris pH 7.0, 500 mM NaCl, and 10% glycerol and frozen at −80 °C.

Membrane pellets were solubilized by the addition of 225 μg of n-dodecyl-β-D-maltoside (DDM) per gram of membrane. Critically, membranes were always resuspended in a volume of 15 μL of buffer per gram of membrane, such that 22 μg of DDM per gram of membrane was a concentration of 1.5% DDM by volume. Membranes were solubilized with a stir bar for 60 min at 4 °C. Unsolubilized material was removed by ultracentrifugation for 142,000 × g for 20 min, 50 mM Tris pH 7.0, 500 mM NaCl, and 10% glycerol and frozen at −80 °C.

Crystallization and Structure Determination. Crystals were grown at 20 °C by vapor diffusion by mixing 250 nL of 3–4 mM protein and 100 mM of reservoir containing 9–11% (v/v) polyethylene glycol glycerol 3500, 200–350 mM LiSO4, and 100 mM sodium citrate pH 5.6–6.0. Bipartitional crystals with a final size of approximately 200 × 200 × 200 μm were obtained after 2–3 d of crystal growth. The three steps that most improved X-ray diffraction resolution were removing the last 59 C-terminal residues to make the 1–645 construct, switching detergent from DDM to LMNG, and dehydrating crystals. To dehydrate crystals, first the crystals were cryoprotected in a mother liquor containing 20 mM Mes pH 6.5, 100 mM NaSO4, and 0.01% LMNG. Peak fractions were collected and concentrated. A typical yield was approximately 1.5 mg per 1 L of starting media.

Crystals were grown at 20 °C by vapor diffusion by mixing 250 nL of 3–4 mM protein and 100 mM of reservoir containing 9–11% (v/v) polyethylene glycol glycerol 3500, 200–350 mM Li2SO4, and 100 mM sodium citrate pH 5.6–6.0. Bipartitional crystals with a final size of approximately 200 × 200 × 200 μm were obtained after 2–3 d of crystal growth. The three steps that most improved X-ray diffraction resolution were removing the last 59 C-terminal residues to make the 1–645 construct, switching detergent from DDM to LMNG, and dehydrating crystals. To dehydrate crystals, first the crystals were cryoprotected in a mother liquor solution supplemented with 25–30% glycerol. After looping the crystal, dehydration was achieved by holding the loop exposed to air for 10 s before flash-freezing in liquid nitrogen. Each of the three unit cell cell dimensions decreased by approximately 5%. Data were collected at the Advanced Light Source beamline 8.3.1. Datasets were processed by using HKL2000 in space group P42222. Molecular replacement was performed with PHASER and
obtained a single solution when using two search components comprised of the Gate and Core domains of human Band 3 (PDB ID code: 4Y27), which presented generally good agreement with the sequence similarity to ATBor1. Iterative model building in Coot (43) and refinement in Refmac5 (26) gradually improved the model as judged by map quality and R factors. Refmac5 was run with jelly-body refinement (σ = 0.03), and with secondary structural restraints turned on. Because our data are low resolution, three modeling strategies were attempted: the human Band 3 starting solution, a poly-alanine model, and a model comprised of the A. thaliana Bor1 sequence based on the solution of Band 3 and refined by Robetta (54). Judging by map quality and R factors, the Robetta model was the best fit. A multiple sequence alignment of Bor1 and Band 3 with secondary structure elements mapped onto them additionally guided the building and residue assignment (Fig. 52). The Fo-Fc difference density and composite omit maps permitted the building of some, but not all, of the missing loops, which were modeled as poly-alanine. The model yielded a crystallographic R factor of 39.5% and a free R factor of 39.1%. MolProbity evaluation of the Ramachandran plot gave 88.0% in favored regions and 1.8% outliers. The overall MolProbity score of 2.33 is in the 99th percentile among proteins in comparable resolution (45). All structural figures were prepared by using PyMOL (46). For comparisons of Bor1 with either Band 3 or UapA, the Gate domains were superposed (i.e., aligned based on structure, and in a sequence-independent manner) by using PyMol. The comparisons were then aligned with the Gate domains to compare Core domain movements relative to the Gate domains.

Complementation Assay. BOR1 was deleted through one-step integration of knockout cassettes (47), resulting in a strain with the following genotype: MATαalpha leu2 trp1-1 ura3-52 his:GAL1-GAL4 pep4 prb1-1122 bor1 Δ::Kanm. Cells were transformed with the same plasmids used for overexpression of protein, bearing HIS3 for selection and under inducible expression by the Gal1 promoter. The only difference in plasmids used in the experiment was whether it contained the negative control of aquaporin AQP1 or a mutation in BOR1 as indicated in Fig. 4. A single colony was picked and grown overnight in CSM-His media containing 2% raffinose. Ten microliters of cells were plated on CSM-His plates supplemented with 2% raffinose, 0.1% galactose, and 20 mM boric acid. Samples started at OD 0.5 and decreased by fivefold serial dilutions. Results were recorded after 5 d at 30 °C.

ACKNOWLEDGMENTS. We thank J. Holton and G. Meigs for assistance with synchrotron data collection at the Advanced Light Source; and Janet Finer-Moore, Alex Kintzer, Jonny Leano, Yi-Liang Liu, Pawel Dominik, and James Fraser for critical reading of the manuscript. This work was supported by University of California Office of the President; Multicampus Research Programs and Initiatives Grant MR-15-338599; the Program for Breakthrough Biological Research, which is partially funded by the Sandler Foundation, for support of beamline 8.3.1; and National Institutes of Health Grant R37 GM024488. B.T-S. was supported by an Alumni Fellow-sponsored Life Sciences Research Foundation fellowship.