Inorganic phosphate (Pi) is a fundamental and essential element for nucleotide biosynthesis, energy supply, and cellular signaling in living organisms. Human phosphate transporter (hPiT) dysfunction causes numerous diseases, but the molecular mechanism underlying transporters remains elusive. We report the structure of the sodium-dependent phosphate transporter from *Thermotoga maritima* (TmPiT) in complex with sodium and phosphate (TmPiT-Na/Pi) at 2.3-Ångstrom resolution. We reveal that one phosphate and two sodium ions (Pi-2Na) are located at the core of TmPiT and that the third sodium ion (Na$_{core}$) is located near the inner membrane boundary. We propose an elevator-like mechanism for sodium and phosphate transport by TmPiT, with the TmPiT-Na/Pi complex adopting an inward occluded conformation. We found that disease-related hPiT variants carry mutations in the corresponding sodium- and phosphate-binding residues identified in TmPiT. Our three-dimensional structure of TmPiT provides a framework for understanding PiT dysfunction and for future structure-based drug design.

**INTRODUCTION**

Maintaining phosphate (Pi) balance is essential for the growth and development of all organisms, and phosphate transporters are key factors in sustaining phosphate homeostasis in humans, plants, fungi, and bacteria. Solute carriers (SLCs) in humans constitute a group of more than 400 membrane transporters allocated to 65 families based on sequence homology. They transport a variety of solutes, such as inorganic ions, amino acids, and neurotransmitters (1). In humans, Pi is translocated into cells by two major secondary active transporters, i.e., the sodium-dependent phosphate transporter SLC20 (PiT) and SLC34 (NaPi-II) families (2), which prefer monovalent (H$_2$PO$_4^{-}$) and divalent (HPO$_4^{2-}$) phosphate, respectively. These families use both the free energy provided by the transmembrane electric field and the Na concentration gradient to cotransport Pi through the membrane with proposed stoichiometries of 2Na:1Pi or 3Na:1Pi (2). The NaPi-II family mainly functions to regulate renal and intestinal Pi transport and comprises three members, named NaPi-IIa, NaPi-IIb, and NaPi-IIc (3). The NaPi-IIa and NaPi-IIb couple the translocation of three Na ions and one divalent Pi (HPO$_4^{2-}$) in each cycle as electronegative transporters, whereas NaPi-Iic is an electroneutral transporter with a transport stoichiometry of two Na to one divalent Pi (HPO$_4^{2-}$) (2, 3). NaPi-II transporters share a similar transport mechanism, with two opposed reentrant helical hairpins (HP1/HP2) being responsible for Na/Pi entry (4).

Human PiTs (hPiT1 and hPiT2) were initially identified as the cell surface receptors for Glvr and Ram retroviruses (5), and they were then subsequently found to cotransport Pi and Na. PiTs exhibit a preference for monovalent Pi (H$_2$PO$_4^{-}$), with a binding affinity of ~50 μM in a pH range of 6.2 to 6.8, which was shown to be lower at both pH 5.0 and pH 8.0 (6). They are ubiquitously expressed in various organs, including the kidney, liver, and brain (2, 7). Dysfunction of hPiTs causes numerous diseases, including vascular and brain calcification (8–11) and neuropsychiatric disorders (10, 11), but the molecular mechanism underlying these transporters remains elusive.

PiTs are present in all kingdoms, including sodium-dependent PiT from *Saccharomyces cerevisiae* ScPiO89 (12), liverwort *Marchantia polymorpha* MpPiT1 (13), and *Plasmodium falciparum* PfPiT (14) or proton-dependent PiT from *Arabidopsis thaliana* AtPiT2 (15) and *Escherichia coli* EcPiTα (16). These PiT proteins have a transmembrane region at both termini linked by a loop or an additional intracellular soluble domain (S domain) in the cellular region (>200 residues) (fig. S1) (17, 18). The two terminal transmembrane regions contain a unique sequence of ~150 amino acids known as ProDom domain 001131 (PD001131), which harbors four highly conserved sequences: GΦNDΦ, GxxxGxxVxxT, ΦΦxxT, and IxxxWΦ (x, any amino acid; Φ, hydrophobic residue) (18–20).

*Thermotoga maritima* is a hyperthermophilic bacterium that makes it an ideal model organism for integration of biochemical and structural experimental approaches (21). We show that sodium-dependent PiT from *T. maritima* (TmPiT) belongs to the SLC20 family, and its transmembrane domain exhibits similar protein characteristics (20) and sequence homology to that of the hPiTs (hPiT1, 62% similarity and 38% identity; hPiT2, 61% similarity and 39% identity) (fig. S1). Structural information for a sodium-dependent PiT had been unavailable. Here, we report the crystal structure of TmPiT, a homology of hPiT. We determined the phosphate-binding affinity and uptake ability of TmPiT. Meanwhile, we solved the crystal structure of TmPiT bound to sodium and phosphate (TmPiT-Na/Pi) at 2.3 Å. The TmPiT-Na/Pi complex is in an inward occluded state and exits as a dimer, with the two subunits showing different conformations.

**RESULTS**

**Crystal structure of the TmPiT-Na/Pi complex**

We overexpressed TmPiT in *S. cerevisiae* and performed x-ray crystallography to determine its structure. In the TmPiT-Na/Pi complex, the transport and scaffold domains are formed by 12 transmembrane
(TM) helices (Fig. 1, A and B). The transport domain of TmPiT adopts a five-helix “5 + 5” fold and is arranged into two inverted PD001131 repeats, i.e., N-PD001131 (TM1/TM2/HP1a-HP1b/TM3) and C-PD001131 (TM6/TM7/HP2a-HP2b/TM8) (Fig. S2A). This five-helix arrangement is distinct from that of the leucine transporter superfamily (22). HP1a-HP1b (HP1) and HP2a-HP2b (HP2) are reentrant helical hairpins that have been reported previously in the aspartate transporter Gltph (SLC1) (23) and the dicarboxylate transporter VcINDY (SLC13) (24) (Fig. 1A and fig. S2A). The four conserved motifs in the two PD001131 repeats are equally distributed to five helices, and all of them are well aligned structurally (Fig. 1 and fig. S2B). TmPiT is a dimer with dimensions of 83 Å by 61 Å by 55 Å, with the N and C termini being located in the extracellular region (Fig. 1C). The dimer interface between the two subunits (A and B) is formed by TM2/TM7 from the transport domain and TM4/TM5 from the scaffold domain through hydrophobic interactions (table S2 and fig. S2D), and this interface has a buried area of 1283.7 Å². The electrostatic surface potential of the Pi-binding pocket is shown in Fig. 1E.

**TmPiT is a sodium-dependent, high-affinity PiT**

Next, we studied the substrate specificity and inhibition of TmPiT. We used microscale thermophoresis to measure the phosphate-binding affinity (Kd) of TmPiT, which was 57.0 ± 1.1 μM (Fig. 2A). The ligand selectivity of TmPiT was investigated using the phosphate analogs arsenate and sulfate, and we calculated a Kd of 6.3 ± 1.0 mM for the former, and there was no apparent interaction with the latter (Fig. 2A), revealing the specific phosphate binding of TmPiT. We also evaluated inhibition of TmPiT by phosphonomiformic acid (PFA), a specific inhibitor of NaPi-II that does not inhibit Pi transport by PiTs (6, 25). As expected, we observed limited binding of PFA to TmPiT, with a calculated Kd of 2.4 ± 1.0 mM (Fig. 2A), supporting that these two types of transporters have distinct binding environments. We further investigated the driving force for phosphate transport in TmPiT using a proteoliposomal Pi uptake assay. In the presence of 120 mM NaCl, Pi uptake was 11.7 ± 0.2 μmol/mg. In the absence of NaCl, Pi uptake was abolished (Fig. 2B), confirming that phosphate transport by TmPiT is driven by sodium.

**Na/Pi binding**

To further understand the mechanism underlying sodium-driven phosphate transport by TmPiT, we analyzed the TmPiT-Na/Pi crystal structure to identify the main residues involved in Pi and Na binding. We observed one phosphate and three sodium ions in our crystal structure of the TmPiT-Na/Pi complex; two of the sodium ions and...
The phosphate (hereafter Pi-2Na) were located at the core of TmPiT (Fig. 2C), and the third sodium (termed Na_{fore}) was situated near the inner membrane (Fig. 2C). Na_{fore} might bind TmPiT first and reorientate TM1 and TM6 into the correct conformation to promote Pi binding (Figs. 2C and 3A). Therefore, we suggest that Na1 and Na2 act as escorts for phosphate binding and Na_{fore} might regulate the binding.

**Conformational changes**

In the TmPiT-Na/Pi complex, we observed notable structural differences between subunits A and B, which are reflected by a root mean square deviation of 1.8 Å for the Cα atoms (fig. S2C), mainly attributable to TM8 and the intracellular loops L7 and LHP2 (Fig. 3, D and E). The accessible volumes of this region, calculated using CASTp (Computed Atlas of Surface Topography of proteins) (27), are 68 and 252 Å³ for subunits A and B, respectively (Fig. 3, D and E, and fig. S3). In subunit A, L7 is within a loosely structured omega loop inside the protein, and Na1 directly interacts with the semiconserved residue K314 (Figs. 2D and 3D and fig. S1). However, in subunit B, L7 is within a three-turn helix that exposes K314 to the solvent, and the interaction with Na1 is eliminated (Fig. 3E). LHP2 in subunit B is longer and more disordered than in subunit A, because there is one less helix turn in HP2b, thereby providing a larger space for Na/Pi release. LHP2 represents the loop that links HP2b and TM8, so helix length and the flexibility of LHP2 may affect TM8 conformation.

Furthermore, there is a notable structural difference between subunits A and B at TM8, reflected by a helix kink in subunit B involving the highly conserved tryptophan W378 that is located in the middle of TM8 and has a distinct orientation (Fig. 3, D and E, and fig. S3).
DISCUSSION

The phosphate-binding mode of TmPiT is unique due to the symmetric repeated residues, which are unlike those of other phosphate transporters, such as E. coli glycerol-3-phosphate transporter (GlpT) (29) and a fungal phosphate transporter (PiPT) (30). GlpT [Protein Data Bank (PDB) ID: 1PWT] is a phosphate antiporter of the SLC37 family that couples the accumulation of sugar phosphates to the downhill release of phosphate under physiological conditions but is not primarily responsible for net phosphate movement (29). PiPT (PDB ID: 4J05) is a Pi\(^{\text{3+}}\)/symporter in the fungus Piriformospora indica. It is a homolog of human SLC22, which functions as an organic anion and cation transporter (30), and presents low sequence homology to TmPiT. Structures of both GlpT and PiPT reveal folding typical of the major facilitator superfamily, with a rocker-switch transport mechanism. In GlpT, one histidine and two arginine residues are proposed to be involved in the phosphate binding (29). In PiPT, the phosphate is buried at the domain interface and coordinated by the aromatic residues Y150, W320, and Y328 and the polar residues Q177, D324, and N431 (30). However, in TmPiT, the Pi-2Na is trapped in a symmetric and hydrophilic environment and bound by D22/S105/T106/T107 and D258/S345/T346/T347. Thus, TmPiT shows a distinct phosphate-binding environment.

In human, both PiT and NaPi-II are sodium-dependent phosphate transporters. When the TmPiT-Na/Pi crystal structure was compared with the modeled NaPi-II (4, 31), we found that the transmembrane domains of PiT and NaPi-II proteins exhibit similar secondary structure features, especially with regard to a common inverted repeat topology. Both structures are capable of binding three Na ions. In TmPiT, Pi-2Na is trapped in a symmetric and hydrophilic environment by two pairs of D/S/T/T motifs (Fig. 2D). For SLC34 protein, corresponding to functional motifs are “161QSSS” and “417QSSS,” which represent the serine-rich stretches of the HPa-HPb linker (4, 31). Structural modeling of NaPi-IIa predicted that the two QSSS motifs are involved in binding Na (Na2 and Na3) and Pi (corresponding to the Na1-Pi-Na2 in TmPiT) (4, 31). Moreover, a conserved aspartic acid is essential for sodium binding, i.e., D327 for Na\(_{\text{core}}\) binding in TmPiT and D224 for Na1 binding by NaPi-IIb. Because of the 2Na1Pi stoichiometry and electrogenicity for PiT, where only two Na ions are transported, it is similar to the electroneutral NaPi-IIC in that two of three Na ions are released (2, 3). This finding suggests that PiT and NaPi-II share functional features despite of significant sequence dissimilarity.

TmPiT (SLC20) shares some structural and functional features with substrate/ion transporters such as the glutamate/Na transporter Glph (SLC1) (23), the dicarboxylate/Na transporter VcINDY (SLC13) (24), and the modeled phosphate/Na transporter NaPi-II (SLC34) (31). Although these sodium-driven transporters do not exhibit sequence homology, they contain transporter domains with two inverted

W378 (TM8) and W139 (TM3) are the corresponding residues in the two inverted repeats, C-PD001131 and N-PD001131, respectively (Fig. 1D). They are highly conserved in the SLC20 family and belong to the IxxxW\(\Phi\) motif. In our mutational studies, both the W139A and W378A mutants lost some Pi-binding ability (Fig. 3F). Patients with primary familial brain calcification disease carry a novel duplication of 626WFVT in hPiT, which corresponds to 378WLLI in TmPiT (28). This mutation does not affect expression of hPiT, but it does alter subcellular protein localization and impairs Pi internalization (28).
repeated helical hairpins (HP1 and HP2) that perform substrate/Na transport, and they have a stable scaffold domain for oligomerization. Substrate/Na transport in these transporters also operates via an elevator-like mechanism (23, 24, 31).

On the basis of the binding and release of substrates/ions and the closing/opening of inner/outer gates, two gate mechanisms were proposed in GltPh (23). Conformational changes in the TmPiT-Na/Pi complex in loops L7/LHP2, and TM8 between subunits A and B indicate that the subunits may have different functional states. Accordingly, these conformational changes may control the inner gate during phosphate and sodium release by assuming closed or open states in subunits A and B, respectively (Fig. 3, D and E, and fig. S3). Residues W139 and W378 are pointed outward and inward, respectively, and may be involved in the opening and closing of the gates. Moreover, subunits A and B reveal different conformations forming an asymmetric dimer, which may imply a cooperative mechanism relationship between the two subunits in TmPiT. However, more experiments and evidence are needed to support this possibility.

On the basis of the sequence repeats in TmPiT, N-PD001131 and C-PD001131 (Fig. 1D); the inverted structure we observed in the TmPiT-Na/Pi complex, TM1-TM2-HP1-TM3 and TM6-TM7-HP2-TM8 (Fig. 1B); and our functional studies of TmPiT mutants revealing that both HP1 and HP2 affect Na/Pi transport; we suggest a two-gated elevator-like mechanism for TmPiT that controls transport at the inner and outer membrane sides, respectively (23). We propose that there might be at least four sequential states, "outward open," "outward occluded," "inward occluded," and "inward open" in the Na/Pi transport cycle (Fig. 4), which occur via a series of conformational changes.

The TmPiT-Na/Pi complex structure is in the occluded state; however, both subunits of the TmPiT dimer reveal different conformations at their inner gates (Fig. 3, D and E). For subunit A, Pi-2Na is completely captured by Na1-Pi-Na2 binding, with Na1 being located near the intracellular membrane and the HP1 tip reaching the inner membrane edge, whereas the HP2 tip is distant from the outer membrane (Fig. 2C). Therefore, we suggest that subunit A exists in the inward occluded state, i.e., the elevator-down position. However, near the inner gate of subunit B, important conformational changes occur, during which TM8 bends and forms a large space that may mimic an open-gate conformation to allow Na/Pi release, the L7 becomes exposed to the solvent, and LHP2 becomes disordered, perhaps reflecting "unlocking" of Na1 (Fig. 3E). Therefore, we speculate that subunit B might adopt the inward open state; when the Na/Pi transport gate opens, Pi and two Na (Nafore and Na1) might release into intracellular, and then Na2 may occupy the Na1 position inside the membrane (Fig. 4).

Since there is a mechanistically symmetrical relationship between the HP1 and HP2 repeats (23), we speculated that in the outward conformation, HP2 may undergo a conformational change similar to that of HP1 in the inward state of TmPiT (Fig. 2C and fig. S4) (32). The HP2 tip may move up and reach the outer membrane edge (Fig. 2C and fig. S4) to form the outward state. In the outward open state, the outer gate will be open to interact with Pi-2Na, and Nafore might be prebound with TmPiT. In the outward occluded state, Na2 of Na1-Pi-Na2 is located near the outer membrane, i.e., the elevator-up position (Fig. 4). However, this outward confirmation we speculated needs additional experiments to be proven.

Certain structural characteristics of TmPiT reflected those reported in disease-associated variants of hPiT. Several variants of hPiT2 are associated with neuropsychiatric disorders and primary familial brain calcification (table S4) (10, 11, 19, 20, 26). To understand how these variants might affect hPiT function, we generated topology and structural models of the hPiT2 membrane domain (Fig. 5A and fig. S5A) using Swiss-Model (33) and based on our TmPiT-Na/Pi complex structure and sequence homology (39% identity and 62% similarity in the membrane domain). The intracellular soluble domain (5 domain) of hPiT2 was not modeled because of its low sequence homology and limited secondary structure prediction. The 5 domain may have additional functions independent of phosphate transport, perhaps acting as an external sensor of extracellular phosphate and/or mediating phosphate signaling in the cell (34).

From our modeled structure of hPiT2 (Fig. 5B), we observed that the proposed Pi-2Na-binding site spans the highly conserved residues for Pi binding, D28, D506, S113/G114/T115, and S593/T594/T595, except for G114 (fig. S5A), as well as those for Na binding (N27/D28/I112/T595/K561 and N505/D506/T115/V592/Wat, except for I112 and V592) (fig. S1). We then mapped disease-related hPiT mutations onto our modeled hPiT structure (Fig. 5B and table S4) and found that most reported clinical mutations lie in the transport

Fig. 4. A working model for Na/Pi transport in TmPiT. The proposed elevator-like mechanism includes four sequential states: outward open, outward occluded, inward occluded, and inward open. The TmPiT-Na/Pi complex (this study) exists as the inward occluded state. The structure shows the transport domains of the two inverted repeats (N-PD001131, magenta; C-PD001131, blue), the scaffold domain (gray), and Pi and Na, as well as TM3/8 and the L2/LHP1 and L7/LHP2 loops. "W" represents Trp139 and Trp378 in TM3 and TM8, respectively.
domains (N-PD001131 or C-PD001131), with a few occupying the dimerization or S domains.

In our mutational studies of TmPiT related to hPiT2 clinical variants (Table 1), we observed that the mutants of the Pi-2Na–binding residues D22A and D258A maintained Pi-binding affinities, but both showed Pi uptake defects. Furthermore, the D22/258A double mutant failed to bind Pi. Mutation of the residue responsible for Na<sub>core</sub> binding, D327Q, resulted in an inability to bind Pi. Moreover, the

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**Fig. 5. Topology and homology modeling of hPiT2 and disease-related variants.** (A) The topology model of hPiT2 was created on the basis of TmPiT. hPiT2 variants linked to brain calcification disease are colored and grouped into six categories: Pi-binding (orange), Na-binding (green), N-PD001131 (pink), C-PD001131 (blue), dimer (yellow), and S domain (brown). (B) The variants were mapped onto the modeled hPiT2 structure and are shown as spheres, colored according to the system in (A). Residue numbers for human/Tm are also labeled (see details in table S4). The Pi- and Na-binding sites are indicated with dashed outlines.
W378A and W139A mutants failed to bind Pi (Fig. 3F). Previously, mutational analysis of hPiT2 residue H502 and sequence mapping predicted it to be a critical residue for the transport function (19, 35). In our structure, the residue in TmPiT corresponding to H502 is H252, which forms hydrogen bonds with the Pi–Na2–binding residues D22 and D258 (fig. S5B). Those interactions are likely abolished in the H502Q mutant of hPiT2, potentially indirectly affecting Pi binding to prevent Pi uptake (fig. S5C). These findings highlight how our TmPiT structural data might inform of the molecular mechanisms underlying human diseases associated with mutations of hPiT.

The TmPiT-Na/Pi complex we present here represents a complete structural study of a sodium-dependent PiT. TmPiT contains a phosphate and three sodium ions tightly bound by TM1/6 and HP1/HP2. We report structural differences between subunits A and B near the inner gate, specifically in loops L7/LHP2 and TM8. We propose that TmPiT uses an elevator-like mechanism to transport phosphate and sodium. Our high-resolution three-dimensional structure of TmPiT may help establish therapeutic targets for diseases associated with PiT dysfunction.

**MATERIALS AND METHODS**

**Cloning, expression, and microsome preparation**

Locus Tm0261 of *T. maritima* strain MSB8 encodes a PiT (TmPiT). TmPiT was amplified from the genomic DNA of *T. maritima* MSB8 strain by polymerase chain reaction (PCR) using Pfu DNA polymerase (MdBio Inc.). The PCR primers were designed so that the 5′ primer contained a restriction site for Hind III, and the 3′ primer contained a restriction site for Bam HI. The amplified DNA was digested with Hind III/Bam HI and then ligated into the expression vector pYES2. A restriction site for Bam HI. The amplified DNA was digested with Hind III, and the 3′ primer contained a restriction site for Hind III, specifically in loops L7/LHP2 and TM8. We propose that TmPiT uses an elevator-like mechanism to transport phosphate and sodium. Our high-resolution three-dimensional structure of TmPiT may help establish therapeutic targets for diseases associated with PiT dysfunction.

**Purification**

Purification of wild-type and mutant TmPiT was performed using a modified "hot-solve" method (37). The microsomes were heated at 65°C for 20 min and then solubilized with n-dodecyl-β-D-maltopyranoside (DDM) for an additional 2 hours before being immediately centrifuged at 4000g for 5 min at 4°C. The TmPiT supernatants were collected and cooled on ice for 10 min and then centrifuged for 20 min at 4°C. The protein sample was purified in a Ni-nitrilotriacetic acid (NTA) column. TmPiT was eluted with buffer [25 mM MES (pH 6.5), 3% glycerol, 120 mM NaCl, 500 mM imidazole, and 0.03% DDM]. The Ni-NTA–purified TmPiT was dialyzed overnight against buffer without imidazole [25 mM MES (pH 6.5), 3% glycerol, 120 mM NaCl, and 0.03% DDM] and then subjected to size exclusion chromatography using a Superdex 200 Increase 10/300 GL column (GE Healthcare) (fig. S6, A and B). TmPiT protein without phosphate was collected for Pi-binding measurements and proteoliposome reconstitution.

**Microscale thermophoresis analysis**

The binding affinity of TmPiT for different ligands—phosphate, arsename, sulfate, and PFA—was measured by microscale thermophoresis.
using a Monolith NT.115^® instrument (NanoTemper Technologies, Germany). The binding affinity of TmPiT mutants for phosphate was assessed using a Monolith NT.115 instrument (NanoTemper Technologies, Germany). TmPiT (10 nM) was labeled with RED-tris-NTA dye (NanoTemper Technologies) in 25 mM MES (pH 6.5), 3% glycerol, 120 mM NaCl, and 0.03% DDM. A volume of 10 μl of 10 nM labeled TmPiT was mixed with 10 μl of ligand (concentrations ranging from 3.1 μM to 50 mM) in 25 mM MES (pH 6.5), 120 mM NaCl, 3% glycerol, and 0.03% DDM. The TmPiT-ligand mixture (4 μl) was loaded into capillaries (NanoTemper Technologies), and thermophoresis was measured at 25°C for 20 s with 15 to 35% LED power and thermo-corporated Pi. Radioactivity was determined by liquid scintillation spectrometry. We conducted the uptake measurement experiment to size exclusion chromatography. Crystallization was performed using the hanging drop vapor diffusion method at 20°C over a reservoir of polyethylene glycol 300 (fig. S6D).

Both the native and anomalous dispersion datasets were collected from the Taiwan Photon Source (TPS) 05A beamline at the National Synchrotron Radiation Research Center in Hsinchu, Taiwan. X-ray diffraction data were processed with the HKL2000 program (38). Native TmPiT crystals belonged to the monoclinic space group C2 with the unit cell parameters a = 121.2 Å, b = 112.6 Å, c = 110.7 Å, and β = 119.3° (fig. S6E and table S1). The Matthew’s coefficient was calculated to be 3.643 Å^3/Da, with a solvent content of 56.4% and two subunits per asymmetric unit.

Structural phasing was determined from the Hg(CH_3COO)_2 heavy-atom derivative. The final phase of the resolution extension was calculated by AutoSol using PHENIX (39), resulting in distinguishable protein and solvent regions (fig. S6F). The initial structural model was built using AutoBuild (39), and the programs Coot (40) and PHENIX (39) were used for model building and refinement. Ultimately, residues 1 to 400 of TmPiT were built. X-ray diffraction data collection and structural refinement statistics are shown in table S1. The final structural model had a crystallographic R-factor of 18.8% and a free R-factor of 22.4%. The solvent-accessible surface area was calculated with the program CASTp (27) using a probe radius of 1.4 Å. All structural figures shown in this report were generated using PyMOL (http://pymol.org/).

Supplementary materials
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/32/eabb4024/DC1

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES

1. M. A. Hediger, B. Clémençon, R. E. Burrier, E. A. Bruford, The ABCs of membrane transporters in health and disease (SLC series): Introduction. Mol. Aspects Med. 34, 95–107 (2013).

2. I. C. Forster, N. Hernandez, J. Biber, H. Murer, Phosphate transporters of the SLC20 and SLC34 families. Mol. Aspects Med. 34, 386–395 (2013).

3. M. Levi, E. Gratton, I. C. Forster, N. Hernandez, C. A. Wagner, J. Biber, V. Sorribas, H. Murer, Mechanisms of phosphate transport. Nat. Rev. Nephrol. 15, 482–500 (2019).

4. C. Fenoll-Ferrer, M. Patti, T. Knöpfel, A. Werner, I. C. Forster, L. R. Forrest, Structural fold and binding sites of the human Na+-phosphate cotransporter NaPi-IIb. Biophys. J. 106, 1268–1279 (2014).

5. M. P. Kavanagh, D. G. Miller, W. Zhang, W. Law, S. L. Kozak, D. Kabat, A. D. Miller, Cell-surface receptors for gibbon ape leukemia virus and amphotropic murine retrovirus are inducible sodium-dependent phosphate symporters. Proc. Natl. Acad. Sci. U.S.A. 91, 7071–7075 (1994).

6. S. Ravera, L. V. Virki, H. Murer, I. C. Forster, Deciphering PiT transport kinetics and substrate specificity using electrophysiology and flux measurements. Am. J. Physiol. Cell Physiol. 293, C606–C620 (2007).

7. M. Inden, M. Iniyama, M. Zennami, S. Sekine, A. Hara, M. Yamada, I. Hazumi, The type III transporters (PiT-1 and PiT-2) are the major sodium-dependent phosphate transporters in the mice and human brains. Brain Res. 1637, 128–136 (2016).

8. S. Yamada, E. M. Leaf, J. J. Chia, T. C. Cox, M. Y. Speer, C. M. Giachelli, PiT-2, a type III sodium-dependent phosphate transporter, protects against vascular calcification in mice with chronic kidney disease fed a high-phosphate diets. Kidney Int. 94, 716–727 (2018).

9. X. Li, H.-Y. Yang, C. M. Giachelli, Role of the sodium-dependent phosphate cotransporter, Pit-1, in vascular smooth muscle cell calcification. Circ. Res. 98, 905–912 (2006).

10. R. R. Lemos, E. M. Ramos, A. Legati, G. Nicolais, E. M. Jenkinson, J. H. Livingston, J. Y. Crow, D. Campion, G. Coppola, J. R. Oliveira, Update and mutational analysis of SLCO2A2: A major cause of primary familial brain calcification. Hum. Mutat. 36, 489–495 (2015).

11. C. Wang, Y. Li, L. Shi, J. Ren, M. Patti, T. Wang, J. R. de Oliveira, M.-J. Sobrido, B. Quintáns, M. Baquero, X. Cui, X.-Y. Zhang, L. Wang, H. Xu, J. Wang, J. Yao, X. Dai, J. Liu, Z. Zhang, H. Ma, Y. Gao, X. Ma, S. Feng, M. Liu, Q. K. Wang, I. C. Forster, X. Zhang, J.-Y. Liu, Mutations in SLCO2A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. Nat. Genet. 44, 254–256 (2012).

12. P. Sengottayai, L. Ruiz-Pavón, B. L. Persson, Functional expression, purification and reconstitution of the recombinant phosphate transporter Pho89 of Saccharomyces cerevisiae. FEBS J. 280, 965–975 (2013).

13. C. Bonnot, H. Prout, B. Pinson, F. P. Colbalchini, A. Lesly-Veillard, H. Breuninger, C. Champion, A. J. Hetherington, S. Kelly, L. Dolan, Functional PTB phosphate receptor binding site model. measurements were combined and analyzed using MO Affinity Analysis 60% microscale thermophoresis power. Data from three independent thermophoresis was measured at 25°C for 20 s with 15 to 35% LED power and loaded into capillaries (NanoTemper Technologies), and thermo-corporated Pi. Radioactivity was determined by liquid scintillation spectrometry. We conducted the uptake measurement experiment to size exclusion chromatography. Crystallization was performed using the hanging drop vapor diffusion method at 20°C over a reservoir of polyethylene glycol 300 (fig. S6D).

Both the native and anomalous dispersion datasets were collected from the Taiwan Photon Source (TPS) 05A beamline at the National Synchrotron Radiation Research Center in Hsinchu, Taiwan. X-ray diffraction data were processed with the HKL2000 program (38). Native TmPiT crystals belonged to the monoclinic space group C2 with the unit cell parameters a = 121.2 Å, b = 112.6 Å, c = 110.7 Å, and β = 119.3° (fig. S6E and table S1). The Matthew’s coefficient was calculated to be 3.643 Å^3/Da, with a solvent content of 56.4% and two subunits per asymmetric unit.

Structural phasing was determined from the Hg(CH_3COO)_2 heavy-atom derivative. The final phase of the resolution extension was calculated by AutoSol using PHENIX (39), resulting in distinguishable protein and solvent regions (fig. S6F). The initial structural model was built using AutoBuild (39), and the programs Coot (40) and PHENIX (39) were used for model building and refinement. Ultimately, residues 1 to 400 of TmPiT were built. X-ray diffraction data collection and structural refinement statistics are shown in table S1. The final structural model had a crystallographic R-factor of 18.8% and a free R-factor of 22.4%. The solvent-accessible surface area was calculated with the program CASTp (27) using a probe radius of 1.4 Å. All structural figures shown in this report were generated using PyMOL (http://pymol.org/).
transporters are present in streptophyte algae and early diverging land plants. New
14. K. J. Saliba, R. E. Martin, A. Bröer, R. I. Henny, C. S. McCarthy, M. J. Downie, R. J. Allen,
A. Mullin, G. I. McFadden, S. Bröer, K. Kirk, Sodium-dependent uptake of inorganic
phosphate by the intracellular malaria parasite. Nature 443, 582–585 (2006).
15. P. Daram, S. Brunner, C. Rausch, C. Steiner, N. Amrhein, M. Bucher, Phi2 encodes a
low-affinity phosphate transporter from Arabidopsis. Plant Cell 11, 2153–2166 (1999).
16. R. M. Harris, D. C. Webb, S. M. Howitt, G. B. Cox. Characterization of PiTα and PiTβ
from Escherichia coli. J. Bacteriol. 183, 5008–5014 (2001).
17. K. B. Farrell, G. E. Tusnady, M. V. Eiden, New structural arrangement of the extracellular
regions of the phosphate transporter SLCO2A1, the receptor for gibbon ape leukemia virus.
J. Biol. Chem. 284, 29979–29987 (2009).
18. C. Salaün, P. Rodrigues, J. M. Heard, Transmembrane topology of PiT-2, a phosphate
transporter-retrovirus receptor. J. Virol. 75, 5584–5592 (2001).
19. P. Bøttger, L. Pedersen, Mapping of the minimal inorganic phosphate transporting unit
of human PiT2 suggests a structure universal to PiT-related proteins from all kingdoms
of life. BMC Biochem. 12, 21 (2011).
20. P. Bøttger, L. Pedersen, Evolutionary and experimental analyses of inorganic phosphate
transporter PiT family reveals two related signature sequences harvesting highly
conserved aspartic acids critical for sodium-dependent phosphate transport function
of human PiT2. FEBS J. 272, 3060–3074 (2005).
21. Y. Zhang, I. Thiele, D. Weekes, Z. Li, J. Zhao, J. Liang, CASTp 3.0: Computed atlas of surface
topography of proteins. Nature 431, 811–818 (2004).
22. R. Mancuso, G. G. Gregorio, Q. Liu, D.-N. Wang, Structure and mechanism of a bacterial
sodium-dependent dicarboxylate transporter. Nature 491, 622–626 (2012).
23. R. Villa-Bellosta, Y. E. Bogaert, M. Levi, V. Sorribas, Characterization of phosphate
transport in rat vascular smooth muscle cells: Implications for vascular calcification.
Arterioscl. Thromb. Vasc. Biol. 27, 1030–1036 (2007).
24. P. Bøttger, L. Pedersen, Two highly conserved glutamate residues critical for type III
sodium-dependent phosphate transport revealed by uncoupling transport function from
retroviral receptor function. J. Biol. Chem. 277, 42741–42747 (2002).
25. W. Tian, C. Chen, X. Lei, J. Zhao, J. Liang, CASTp 3.0: Computed atlas of surface
topography of proteins. Nucleic Acids Res. 46, W363–W367 (2018).
26. I. Taglia, P. Formichi, C. Battisti, G. Peppoloni, M. Barghigiani, A. Tessa, A. Federico,
Primary familial brain calcification with a novel SLC20A2 mutation: Analysis of PiT-2
expression and localization. J. Cell. Physiol. 233, 2324–2331 (2018).
27. Y. Huang, M. J. Lemieux, J. Song, M. Auer, D.-N. Wang, Structure and mechanism of the
glycerol-3-phosphate transporter from Escherichia coli. Science 301, 616–620 (2003).
28. B. P. Pedersen, H. Kumar, A. B. Wright, A. J. Risenmay, Z. Roe-Zurz, B. H. Chau,
A. Schlessinger, M. Bonomi, W. Harries, A. Sali, A. K. Johri, R. M. Stroud, Crystal structure
of a eukaryotic phosphate transporter. Nature 496, 533–536 (2013).
29. C. Fenoll-Ferrer, L. R. Forrest, Structural models of the NaPi-2 sodium-phosphate
cotransporters. Pflugers Arch. 471, 43–52 (2019).
30. L. R. Forrest, Y.-W. Zhang, M. T. Jacobs, J. Gesmonde, L. Xie, B. H. Honig, G. Rudnick,
Mechanism for alternating access in neurotransmitter transporters. Proc. Natl. Acad.
Sci. U.S.A. 105, 10338–10343 (2008).
31. A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer,
T. A. P. de Beer, C. Rempfer, L. Bordoli, R. Schwede, SWISS-MODEL: Homology
modelling of protein structures and complexes. Nucleic Acids Res. 46, W296–W303 (2018).
32. N. Bon, G. Coussay, A. Bourdine, S. Sorceuse, S. Beck-Cormier, J. Guicheux, L. Beck,
Phosphate (P)-regulated heterodimerization of the high-affinity sodium-dependent P,
transporters PiT1/Slc20a1 and PiT2/Slc20a2 underlies extracellular Pi sensing
independently of Pi uptake. J. Biol. Chem. 293, 2102–2114 (2018).
33. C. S. Hsu, R. L. Sears, R. R. Lemos, B. Quintâns, A. Huang, E. Spitleri, L. Nevarez, C. Mamah,
M. Zatz, K. D. Pierce, J. M. Fullerton, J. C. Adair, J. E. Berner, M. Bower, H. Brodaty,
O. Carmona, V. Dobricì, L. B. Fogel, D. García-Estevez, J. Goldman, J. L. Goudreau,
S. Hopfer, M. Janković, S. Jaumà, J. C. Jen, S. Kirdlarp, J. Klepper, V. Kostić, A. E. Lang,
A. Linglart, M. K. Maiensbacher, B. V. Manyam, P. Mazzoni, Z. Miedzybrodzka,
M. Mitarnoun, P. B. Mitchell, J. Mueller, I. Novaković, M. Paucar, H. Paulson, S. A. Simpson,
P. Svenningson, P. Tuite, J. Viték, S. Wetchaphanhesat, C. Williams, M. Yang,
P. R. Schofield, J. R. de Oliveira, M. J. Sobirob, D. H. Geschwind, G. Coppola, Mutations in
SLC20A2 are a major cause of familial idiopathic basal ganglia calcification.
Neurogenetics 14, 11–22 (2013).
34. J. Kim, J. Weiss, A. M. van Rhee, T. Schöneberg, K. A. Jacobson, Site-directed mutagenesis
identifies residues involved in ligand recognition in the human α2-adrenergic receptor.
J. Biol. Chem. 270, 13987–13997 (1995).
35. R. L. López-Marqués, J. R. Pérez-Castiñeira, M. J. Buch-Pedersen, S. Marzo, J.-L. Rigaud,
M. G. Palmgren, A. Serrano, Large-scale purification of the proton pumping
pyrophosphatase from Thermotoga maritima: A "hot-solve" method for isolation of
recombinant thermophilic membrane proteins. Biochim. Biophys. Acta 1716, 79–86
(2005).
36. Z. Owinoowski, W. Minor, Processing of x-ray diffraction data collected in oscillation
mode. Methods Enzym. 276, 307–326 (1997).
37. P. D. Adams, P. V. Afonine, G. Bunkóczi, V. B. Chen, I. W. Davis, N. Echols, J. J. Headd,
L.-W. Hung, G. J. Kapral, R. W. Grosse-Kunstleve, A. J. McCoy, N. W. Moriarty, R. Oeffner,
R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger, P. H. Zwart, PHENIX:
A comprehensive Python-based system for macromolecular structure solution.
Acta Crystall. D 66, 213–221 (2010).
38. P. Emmsley, B. Lohkamp, W. G. Scott, K. Cowtan, Features and development of Coot.
Acta Crystall. D 66, 486–501 (2010).

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