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***P. aeruginosa* PilT structures with and without nucleotide reveal a dynamic Type IV pilus retraction motor**

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Abstract

Type IV pili are bacterial extracellular filaments that can be retracted to create force and motility. The retraction is accomplished by the motor protein PilT. Crystal structures of *Pseudomonas aeruginosa* PilT with and without bound AMP-PCP have been solved at 2.6 and 3.1 Å resolution, respectively, revealing an interlocking hexamer formed by the action of a crystallographic 2-fold symmetry operator on three subunits in the asymmetric unit and held together with extensive ionic interactions. The roles of two invariant carboxylates, Asp Box motif Glu163 and Walker B motif Glu204, have been assigned to Mg²⁺ binding and catalysis, respectively. The nucleotide ligands in each of the subunits in the asymmetric unit of the AMP-PCP bound PilT are not equally well ordered. Similarly, the three subunits in the asymmetric unit of both structures exhibit differing relative conformations of the two domains. The 12° and 20° domain rotations indicate motions that occur during the ATP-coupled mechanism of disassembly of pili into membrane-localized pilin monomers. Integrating these observations, we propose a three-state Ready, Active, Release model for the action of PilT.

Keywords

Type IV pili; Motor protein; P-loop ATPase; Crystallography

Introduction

Type IV pili (Tfp) are extracellular appendages present in a wide variety of Gram-negative and some Gram-positive bacteria including pathogens of plant, fungi and animals, soil-dwelling bacteria, and extremophiles^{1; 2}. While not necessary for viability, Tfp play an important role in the lifestyle of many bacteria by participating in biofilm formation, cell adhesion, phage uptake, DNA uptake, and a type of flagellar-independent surface motility often called twitching motility³. Thus, understanding the mechanisms that power pilus assembly and disassembly will yield significant information about how bacteria use pili for virulence and about the mechanisms of molecular motors. Gene products that control Tfp regulation, assembly, and disassembly have been identified and their roles characterized in several model organisms^{1; 4}. The three-dimensional structure of the pilus filament has been

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Accession Codes

The AMP-PCP and unliganded *P. aeruginosa* PilT structures have been deposited in the Protein Databank with codes 3JVV and 3JVU, respectively.

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studied using multiple biophysical techniques⁵ and recent work has begun to elucidate the interactions of conserved membrane proteins at the base of the pilus⁶.

The force generated by the retraction of a single pilus is over 100 pN⁷, and the retraction of a Tfp bundle leads to nN forces⁸. PilT, the homohexameric machinery that powers pilus retraction, is thus the strongest biological motor known. PilT belongs to a family of secretion ATPases that are conserved in the Type IV pili, Type II secretion, and Type IV secretion systems and defined by four signature sequences. The Walker A and B motifs are canonical in P-loop ATPases, a group which includes motor proteins F₁-ATP synthase, myosin, RecA, and many helicases. The Asp and His boxes are motifs unique to the secretion ATPase family. As the name implies, the Asp box contains two conserved carboxylic acid residues. These are likely involved in coordinating the active site geometry. The His box contains two namesake histidine residues, and substituting one of these residues leads to loss of PilT activity *in vivo*^{9; 10}.

The structures of five secretion ATPases are known^{9; 11; 12; 13; 14}. All share a bi-lobed architecture consisting of a PAS-like N-terminal domain (NTD) joined by a flexible linker to a RecA fold C-terminal domain (CTD). The ATP binding site lies between the two domains, with all four of the signature motifs found near the nucleotide in the CTD. We have previously reported the structure of PilT from *Aquifex aeolicus*⁹, a hyperthermophile whose PilT is 51% identical to *P. aeruginosa* PilT. That structure highlighted another invariant feature of PilT and other secretion ATPases; a pair of clamping arginines at the tips of NTD β 5 and β 6, which interact with the phosphates of ATP and drive conformational change in the motor^{9; 14}. In order to understand the detailed steps of pilus retraction we have now solved both the apo and ligand-bound structures of PilT from *P. aeruginosa*, an important and experimentally amenable pathogen with well-characterized Type IV pili.

Results

Tertiary and quaternary structure of AMP-PCP bound PilT

P. aeruginosa PilT (PaPilT) was crystallized in the presence of the non-hydrolyzable ATP analog β,γ -methyleneadenosine 5'-triphosphate (AMP-PCP). The PaPilT structure was solved by molecular replacement using *Aquifex aeolicus* PilT (AaPilT) as a model, and refined to 2.6 Å resolution (Table 1). The structures presented in this paper are the first of a retraction motor with relevance to *in vivo* pathogenicity. Unlike the previously solved AaPilT structures⁹, the PaPilT structure has active sites that are in the correct conformation to bind and hydrolyze nucleotide as well as a sufficiently high resolution to warrant interpretation of side chain and ligand positions. The three subunits in the asymmetric unit (A, B, and C) conform to the two-domain structure (Figure 1a) and application of the crystallographic 2 fold axis forms the biologically relevant hexamer (Figure 1b). As expected each domain maps well onto the AaPilT structure, with r.m.s.d. 1.2 Å or 1.3 Å for superposition of the N- or C-terminal domain, respectively. The NTD (1–98) is a 6-stranded anti-parallel β -sheet flanked on one side by 3 α -helices. The CTD (105–344) is a curved 7-stranded β -sheet sandwiched between α -helices. Strong electron density for the extended β -hairpin between anti-parallel β -strands 12 and 13 is only observed for the C subunit. One of these is the AIRNLIRES helix, which is necessary for pilus retraction *in vivo*¹⁵ (Figure 1a).

PilT forms a closed hexameric toroid in the crystal lattice with an outer diameter of 115 Å and height 60 Å (Figure 1b). The inner pore is 40 Å in diameter at the N-terminal opening and tapers to a 13.1 Å outlet constricted by Glu247, located on a loop between α 9 and α 10. Overall, the hexamer surface displays patches of positive, negative and neutral character including some exposed hydrophobic side chains which are not buried by crystallization (Suppl. Figure 1A). For example, NTD side chain Phe9 is on the outside of the hexameric

torus while Phe66 faces into the lumen (Suppl. Fig. 1A). Extensive subunit interlocking forms the toroid, with each subunit contributing 3,000–3,100 Å² of buried surface area. The major surface (~2,000 Å² total per interface) is CTD^N:NTD^{N+1} packing, including mainly ionic side-chain interactions, few hydrophobic interactions and 2 cation- π interactions (Arg176 of CTD^N to Phe86 NTD^{N+1} and Tyr152 of CTD^N to Arg35 from NTD^{N+1}). The additional CTD:CTD interface contributes ~1,000 Å² total per interface.

A sequence alignment of PaPilT with other secretion NTPases was mapped onto the surface of PilT in order to highlight conserved structural elements across the secretion ATPase family (Figure 2)¹⁶. The most conserved amino acids lie at or near the tripartite ATP active site formed in the cleft between the NTD^N and CTD^N with additional contributing residues from the back side of the adjacent CTD^{N-1} (Figure 2). The least conserved residues are located in the far CTD α -helices, an observation which indicates this area can be used to distinguish PilT from other assembly ATPases, such as PilB, or secretion proteins such as GspE. Only a few of the buried surface contacts are highly conserved among these 27 secretion ATPases (Figure 2).

The active site configuration of the nucleotide-bound PilT

In each tripartite NTD^N:CTD^N:CTD^{N-1} nucleotide binding site, there was strong electron density for an AMP-PCP molecule and a Mg²⁺ ion from the beginning of the refinement process. The presence of these ligands allows us to extend current understanding of the roles of each of the four conserved secretion ATPase motifs, which are known to be essential for function^{9; 10; 15}. The canonical Walker A phosphate (P-) loop residue Lys136 is pointed directly at the γ -phosphate, coordinating the ligand at a distance of 2.8 – 3.6 Å (Figure 3a). The backbone nitrogens of the P-loop coordinate the β - and γ -phosphates. Walker A residue Ser137 makes a short interaction with the Mg²⁺ ion.

In all three subunits, carboxylic acid oxygens of the Asp Box Glu163 interact directly with the well-ordered Mg²⁺ ion (Figure 3a). No other carboxylate side chain is in a position to fulfill this role, thus conclusively demonstrating the Mg²⁺ binding role of this Asp Box residue and eliminating the possibility that a side chain from the nebulous Walker B motif is directly involved in Mg²⁺ binding. In our earlier *A. aeolicus* PilT structure, the proximity of this residue to the ATP was clear, but we were unable to distinguish between a catalytic role and a Mg²⁺ binding role. Undoubtedly, this assignment of the Asp Box glutamate as a Mg²⁺ interacting residue is also appropriate for other secretion ATPases¹⁴.

The role of the long Walker B motif, which traverses the entire CTD, is not well understood for the secretion ATPases, and no results are available that suggest a role other than a structural one for the first ten amino acids of this 13 amino acid sequence. Surprisingly, weak density for the last four side chains of the Walker B motif, those closest to the nucleotide, indicates they are disordered, potentially because of solvent exposure and/or the non-biological substrate in our crystals. Nonetheless, the active site carboxylic acid closest to the γ -phosphate and in an appropriate orientation to attack and activate a water molecule close to the γ -phosphate is residue Glu204. Substitution of this side chain abolishes ATPase activity¹⁰ and we would predict this effect is largely due to an effect on k_{cat} .

The two His Box histidines from a single subunit are not near each other in three dimensions. Although His229 approaches the γ -phosphate of the nucleotide in the binding pocket of the subunit in which it is found (3.9 Å away), His222 is closer to the nucleotide from the adjacent subunit (8.4 Å from the γ -phosphate) (Figure 3b). These two histidines are 8.1 Å from each other across the subunit interface. Indeed, a pair of ionic interactions from His229^N to Thr220^{N-1} and from His222^{N-1} to Thr132^N (part of the P-loop) do make a three-dimensional Histidine Box across the CTD^N:CTD^{N-1} interface (Figure 3b).

The three ligand binding sites are not equivalent

Several conserved residues in the active site vary in conformation between subunits, with subunit B most poised to hydrolyze ATP. The greatest differences are found in the NTD clamp arginines, Arg82 and Arg97, which coordinate the γ -phosphate. Substituting either of these residues leads to an abrogation of PilT function *in vivo*⁹. In subunit A, Arg82 and Arg97 approach the γ -phosphate (5.0 and 5.2 Å respectively) (Figure 4a). In subunit B, they are locked onto the nucleotide. Arg82 makes two salt bridges to the γ -phosphate (3.1 and 3.3 Å) and Arg97 is 4.3 Å away (Figure 4b). In subunit C the clamp arginines are swung away from the nucleotide at distances of 9.0 and 4.5 Å (Figure 4c).

His229 is further from the γ -phosphate in subunits A and C (4.5 or 4.4 Å) than in subunit B (3.9 Å). The close approach in subunit B could be part of a His Box mediated intersubunit communication, and is consistent with a proposed role as a phosphate sensor¹⁰. In addition to coordination across subunits, this closely-approaching His229 might contribute directly to the catalytic hydrolysis of ATP, as has been proposed for the “lynchpin Histidine” of the ABC transporter family¹⁷. Similarly, the carboxylate oxygens of Glu163 are further from the Mg^{2+} ion in subunits A and C (3.0 and 3.4 Å; 3.1 and 4.1 Å) than in subunit B (3.0 and 3.0 Å).

The ligands themselves have noteworthy differences among the subunits. A, B and C nucleotide and Mg^{2+} have accessible surface areas of 109.9, 71.7 and 91.5 Å² and average B-values assuming full occupancy of 70.4, 60.9, 70.7 Å², respectively. Thus, the ligands of subunit B are the most engaged inside the binding cleft. The clamp arginines in subunit C are swung away from the AMP-PCP, suggesting that they guide inorganic phosphate away from the ATP binding site towards the lumen of PilT. The hydrolyzed nucleotide can diffuse out of the highly solvent accessible active site, the same way it likely came in (Suppl. Fig. 1a).

Key differences between nucleotide bound and unliganded PilT structures

To assess the potential differences between nucleotide-bound and unliganded forms of the pilus retraction motor, PilT was purified and crystallized in the absence of nucleotide and Mg^{2+} , yielding crystals under similar conditions and of the same space group and unit cell as the AMP-PCP form. Comparing the nucleotide-binding sites of the AMP-PCP bound B subunit and the Apo-PilT B subunit identifies key functional residues (Figure 3). The clamp residues Arg82 and Arg97 vary most between the two structures. Glu163 has also changed positions in the unliganded structure presumably due to the lack of Mg^{2+} . The conformation of the P-loop and positioning of the catalytic Lys136 remain similar. His222 within the 3D His Box undergoes a conformational twist of 30° in A, 45° in B, and 0° in C. The β 12- β 13 hairpin that shelters the adenine moiety (Fig. 4a-c) is, paradoxically, less well-ordered in the nucleotide-bound than in the apo structure.

The unliganded hexamer has similar domain conformations to the AMP-PCP bound PilT toroid and the same overall outer dimensions. The calculated R_g is unchanged (34.6 Å for the Apo PaPilT hexamer and 34.5 Å for the AMP-PCP PaPilT hexamer¹⁸). To a first approximation, the openness of the PilT subunits is thus an inherent property of the hexameric structure rather than a consequence of nucleotide binding. At a more detailed level, local structural changes are manifest that could be significant for ATP hydrolysis and subunit:subunit signaling. For example, in the AMP-PCP bound subunit B, Glu336 of the far C-terminal α 12 is well-ordered and forms a salt bridge to Arg123 in the CTD of neighboring subunit A. However, α 12 is not visible in the apo subunit B.

A striking difference between the two structures is the conformation of the central pore. The lumen opening is more oblong in the unliganded structure, with the already limited opening

decreased. The distance from subunit A Glu247 main chain nitrogen to its symmetry mate across the lumen is 15.7 Å in ligand-bound PilT vs 13.2 Å in the apo case. Glu247 side chains are also closer to the center of the hexamer in the apo structure, such that the overall constriction decreases from 13.1 to 5.8 Å. This happens as $\alpha 9$ and $\alpha 10$ are translated toward the center along their own helical axes by about 1.5 Å (refer to Figure 1b).

Direction of force generated by PilT nucleotide binding

To further investigate the conformational changes of PilT that might be important for motor function, we compared relative domain orientations among chains A, B, and C¹⁹. While the sequences of the three subunits are identical, the structures are not. Subunit A is the most open, B is intermediate, and C is the most closed (Figure 4d). Accordingly, the domain motion required to move the A subunit NTD into a position equivalent to the C subunit conformation is 19.6° (Fig. 5a). The intermediate position of B's NTD is on the trajectory from A to C, with an 11.7° rotation from A to B and an additional 9.4° rotation from B to C. The magnitude of these motions is on par with that observed in crystal structures of other secretion NTPases, such as GspE (PDB code 2OAP) which has a 21.8° rotation¹⁴ (Fig. 5b). PaPilT does not have the 70° domain rotations observed in the AaPilT structure, which may have been a result of crystal packing forces⁹.

It has been postulated that within hexameric rings of the RecA superfamily of proteins the direction of force generation can be predicted relative to the azimuthal axis of the ring based on the orientation of the central β -strands of the RecA domain. In F₁-ATPase and HslU, it was noted that the force vector that connects the centers of masses of the moving domains when the RecA domains are aligned and kept constant parallels the β -strands of the RecA domain²⁰. F₁-ATPase and HslU undergo domain motions of 30° and 20°, respectively, upon binding of nucleotide^{21; 22; 23}. The F₁-ATPase β -strands are oriented nearly perpendicularly to the azimuthal ring axis, with the plane of the β sheet matching the flat plane of the hexamer, and the direction of force generation is also within this plane (Fig. 5d). The resulting motion spins the central stalk of the rotary motor in the plane of the membrane. HslU's canonical RecA β -strands are oriented almost parallel to the ring axis, and HslU generates force parallel to the ring axis (Fig. 5d), as needed for its role in pushing polypeptide chains through its central pore and delivering them to be degraded by the HslV protease²⁴. The RecA domain β -strands of the PilT subunits are arrayed in the third possible orthogonal orientation; the strands are again perpendicular to the ring axis but unlike F₁-ATPase the β sheet climbs along the azimuthal axis (Figure 5a). This is also the case for secretion ATPases GspE and HP0525 (Fig. 5b,c). The force vector observed for these secretion ATPases when keeping superimposed RecA domains fixed and aligning the NTDs is diagonal to the ring axis. This diagonal force generation during the closing of the NTD onto the nucleotide causes the NTD to push on the CTD of the adjacent subunit (Supplementary movie S1). Although this motion does not follow the elementary predictive rule, it is indeed the sum of the first two force vectors, and the direction of this vector does match the required movements for the biology. In the simplest model for PilT motion, pilin subunits must be moved coordinately from their position in the pilus filament *down* the central axis toward the membrane and *away* from the axis into the plane of the membrane.

Discussion

Ready, Active, Release Motor

Based on the differing geometries of the three PilT subunits with respect to the ATP binding site, we propose the "Ready, Active, Release" model for PilT action (Figure 4D). Beginning with the Ready or open, unliganded state (represented by subunit A), ATP binding forces the N- and CTDs to approach each other as the clamp arginines interact with the phosphates.

This is the largest conformational change during the PilT motor cycle, and results in the Active conformation for ATP hydrolysis (represented by subunit B). Subunit C represents the Release or closed, post-hydrolysis conformation. Unlike the rotary motor of the F_1F_0 ATPase, which depends on the sequential actions of the Binding Change Model²⁵, we would predict the PilT subunits can act stochastically, as there is no reported rotary motor mechanism. With the simplifying assumption that PilT interacts directly with pilin subunits, one can imagine that any stabilizing interaction that prevents the pilus from slipping backwards while a second motor subunit binds to a subsequent pilin monomer and undergoes ATP-dependent domain rotation would lead to retraction. This model does not preclude sequential binding as the most efficient mode of action of the motor, possibly generating greatest forces and/or speed²⁶. A recent study on the dynamics of Tfp retraction by Clausen and coworkers showed that pilus retraction velocity is bimodal, and that the elementary steps of pilus retraction are less than 3 nm and therefore potentially correspond to a single pilin subunit^{26; 27}. The net displacement of the PaPilT NTD during the domain rotation from the Ready to the Active subunit conformation is on the order of 0.8–1 nm, and the rise between pilin subunits in an assembled Type IV pilus is similarly on the order of 1 nm⁵. If the interaction between PilT and pilin is direct, then Clausen and coworkers' conclusion is consistent with 1 ATP hydrolysis event by PilT leading to the disassembly of 1 pilin subunit. How might the pilus retraction motor be able to function in a low velocity and a high velocity mode? One possibility is that the high velocity mode comes from coordinated PilT activity in which all six subunits are engaged and presumably firing sequentially around the hexamer while the low velocity mode is the result of a random firing of PilT subunits. There is precedent in other ATP-driven machines, the ClpX protein translocation motor and the GroEL chaperone, to function when only a subset of the subunits have native ATP or protein binding sites, respectively^{28; 29}.

Potential mechanism of PilT-driven pilus disassembly

How PilT couples large domain motions to type IV pilus retraction is a key question. PilT utilizes its NTD to localize to the inner membrane³⁰. While hydrophobic residues are typically not found on the surface of a protein, conserved Phe9 and (non-conserved) Phe66 are surface exposed residues that could be involved in interactions with lipids, an inner membrane protein dock and/or pilin itself. Pilin subunits cannot disassemble by passage through PilT, as the opening at the base is not large enough to accommodate a pilus filament nor is it logical to expect an entire pilus to retract into the cytoplasm. Rather, pilus retraction is a reversible disassembly process resulting in membrane-spanning pilin monomers.

A direct model in which PilT engages pilin α -helical tails and shuttles subunits into the inner membrane along a vector that follows the diagonal path from the base of the filament to the inner membrane is conceivable, and the relative sizes of PaPilT and full length pilin subunits are appropriate for this model. The change in ligand-bound vs. apo PilT lumen diameter suggests the lumen could change conformation to accommodate pilin tails during retraction. We hypothesize a paddle-wheel mechanism for retraction, in which the PilT lumen loops or NTD structural elements engage the exposed helical N-terminal tail of pilin (Fig. 6a), and pull on it directly as the Open subunit NTD swings toward the neighboring CTD upon ATP binding and transition to the Active state. This model is consistent with a growing appreciation that other molecular machines including SecY, HslU, and ClpX use a paddle-wheel mechanism to push or pull unfolded proteins through protein channels or across membranes^{31; 32; 33}. There are serious disadvantages to this direct interaction model, though, including no demonstrated interaction between pilin and PilT, and the likelihood that the pilin N-terminal helix does not extend far enough across the inner membrane to interact extensively with PilT (Fig. 6a).

Given the high conservation of proteins required for Tfp assembly and retraction, a model in which PilT interacts directly with a conserved inner membrane platform protein (IMPP) such as PilN, PilO, or PilC (Fig. 6b) is a likely alternative to the direct interaction model. In such a scenario, PilT's action on the IMPP would alter the relative orientation of this protein with the lowest pilin subunits and the IMPP would act on pilin to remove it from the filament and translate it into the membrane. Recent results describing a stable PilN:PilO heterodimer with a coiled-coil region in the periplasm⁶ hint at a mechanism in which a short conserved cytoplasmic region of PilN interacts directly with PilT, and force is transmitted across a long transmembrane helix and coiled-coil lever arm to the periplasm, where PilN:PilO could act directly on pilin subunits. A scissoring motion of PilN and O could be readily reversible and transmit force signals over long distances. It is also possible that the multipass inner membrane protein PilC plays a role in retraction, as its homolog in the enteropathogenic *E. coli* type IVb Bundle Forming Pilus pathway interacts with the two secretion ATPases in that system³⁴, although PilC and its homologues in Tfp-a systems behave differently than those in the Tfp-b³⁵. In this scenario, PilC's large cytoplasmic N-terminal region would interact with PilT (potentially with the AIRNLIRE motif) and force would be transmitted through this domain via PilC's inner membrane helices to pilin's N-terminal membrane helix to act as a wrench or pliers to remove the bottom-most pilin subunit from the filament. The action of short helical hairpins within the lipid bilayer during uptake by the glutamate transporter³⁶ suggests there could be a function for short hydrophobic stretches not long enough to be complete transmembrane helices in the predicted membrane topology for PilC.

Materials and Methods

Protein expression and purification

The *pilT* gene was cloned from *P. aeruginosa* PA103 genomic DNA using the primers 5'-GGGAATTCCATATGGATATTACCGAGCTGCTCGCCT-3' and 5'-CCTTTGCGGCCGCTCAGAAGTTTTCCGGGATCTTCGCCTTC-3' engineered with *NdeI* and *NotI* sites for cloning into a pET23a expression vector (Novagen). The primers 5'-CCCGGAAAACTTCGGATCCTGGCGCCGATCCGCCGCGC-3' and 5'-GCGCGGCGGATCGGCGCCAGGATCCGAAGTTTTCCGG-3' were used to change the stop codon to read through the C-terminal His tag by site directed mutagenesis to produce the final expression plasmid (pET23a-PaPilT-His). The cloning was verified by automated DNA sequencing (University of Wisconsin – Madison, DNA Biotechnology Sequencing Center).

For protein expression, pET-23a-PaPilT-His was transformed into BL21(DE3)pLysS chemically competent *E. coli* cells (Promega). Cells were grown at 37°C with shaking at 220 rpm in Luria-Bertani growth medium supplemented with 100 µg/mL ampicillin. The culture was induced with 1 mM IPTG at an OD₆₀₀ of 0.6 and the temperature was dropped to 20°C for overnight protein expression. The following morning, the cells were harvested with a 15 minute 3,000 g centrifugation and pellets were stored at -80°C until ready for use. For purification, the pellet from 1 L of bacterial culture was freeze-thawed and resuspended in 30 mL Buffer A (50 mM Tris, 1 M sodium chloride, 5% glycerol, 40 mM imidazole, pH 7.1). The cells were lysed by sonication at 30% power for 2 minutes (15 seconds on/15 seconds off) pulses. The lysate was cleared with centrifugation at 76,000 g for 30 minutes. The cleared lysate was added to 2.7 mL of Ni-NTA resin (QIAGEN) and allowed to bind for 1 hour at 4°C with gentle shaking. After batch binding, the slurry was added to an empty column (Bio-Rad) and allowed to filter by gravity. The flow through was discarded, and the column was washed with 10 column volumes of Buffer A, or until no remaining protein came off the column, as assayed by A₂₈₀ absorbance. The protein was eluted with Elution Buffer (50 mM Tris, 5% glycerol, 1 M sodium chloride, 250 mM imidazole, pH 7.1), and

samples were concentrated using a centrifugal filter device (Millipore). Purity and homogeneity were assayed by Coomassie-stained polyacrylamide gel electrophoresis (PAGE) and by dynamic light scattering (Protein Solutions, DynaPro). Conditions for optimum solubility were identified using a solubility screen³⁷, following which samples were dialyzed overnight at 5°C into Buffer 5 (50 mM sodium chloride, 50 mM 2-(N-Morpholino)-ethanesulfonic acid (MES), 50 mM sodium citrate, 5% glycerol, 5 mM magnesium chloride, pH 5.5).

Crystallization

The protein was concentrated to 26 mg/mL as determined by absorbance at 280 nm and using a calculated extinction coefficient³⁸. AMP-PCP, a non-hydrolyzable ATP analog (Sigma), was added to a concentration of 1.4 mM such that the molar ratio of PiIT subunits to nucleotide was 1:2. The protein/nucleotide complex was incubated on ice for 20 minutes prior to setting up a sparse matrix crystallization screen (JSCG - Nextal). An initial crystallization hit was obtained in drops containing 2 μ L protein and 2 μ L mother liquor with 10% PEG 6000 and 100 mM Hepes, pH 7.5 in the well. Crystallization conditions were refined, and crystals measuring $0.4 \times 0.2 \times 0.15$ mm were grown in 7 days at room temperature. Crystals were cryo-preserved with a slow transfer in 6 incremental steps from cryo-protection buffer A (10% PEG 6000, 100 mM HEPES pH 7.5, 50 mM sodium citrate, 50 mM sodium chloride, 10% glycerol, 1.4 mM AMP-PCP, 5 mM magnesium chloride) to cryo-protection buffer B (15% PEG 6000, 100 mM HEPES pH 7.5, 50 mM sodium citrate, 500 mM sodium chloride, 25% glycerol, 1.4 mM AMP-PCP, 5 mM magnesium chloride) over the course of 1 hour. Crystals were flash-cooled in liquid nitrogen. Unliganded PiIT crystals were obtained in a similar method, except that nucleotide and magnesium chloride were not included in the crystallization or cryoprotection solutions.

Data Collection and Processing

A 2.6 Å resolution dataset and a lower resolution shorter exposure sweep were collected on one crystal at the Advanced Photon Source (APS) beamline 14-BM-C on an ADSC Quantum 315 detector. The unliganded PaPiIT dataset was collected at APS at the LS-CAT 21-ID-D beamline on a Mar CCD detector. Each dataset was integrated, scaled and merged with HKL2000 (Otwinowski and Minor, 1997) (Table 1).

Structure determination and refinement

The 2.6 Å resolution structure of full-length nucleotide bound PaPiIT was determined in space group C222₁ with 3 monomers per asymmetric unit. Phases were obtained by molecular replacement with PHASER³⁹ using a non-biological subunit, the CTDⁿ:NTDⁿ⁺¹ domains of *A. aeolicus* PiIT (PDB code 2EWV), as the search model. Manual modeling and addition of waters were conducted in XFIT and Coot⁴⁰. Restrained refinement with isotropic TLS B-factor refinement and non-crystallographic symmetry (NCS) constraints was carried out with REFMAC v5.5⁴¹. NCS sets included NTD residues 1–93 and CTD residues 103–342 and excluded ligands and linker regions. The β -hairpin region 270–277 in chain A, 272–277 in chain B, and loop 320–326 in chain A were not modeled due to poor density in these regions. No electron density for any of the His tags was observed. Interestingly, a strong region of positive $F_o - F_c$ was identified at the crystal packing contacts of two non-crystallographically related hexamers. A citrate ion was refined into this density due to good fit of the size and shape of the density, the positively charged residues surrounding the pocket and the presence of citrate in crystallization conditions (Supplemental Figure 1).

The 3.1 Å resolution structure of full-length unliganded PaPiIT was solved in the same space group, using initial phases derived from molecular replacement using the ligand-bound

PaPilT CTD^N:NTD^{N+1} structure. Initial TLS B-factors were also obtained from the AMP-PCP structure, and Refmac was run using overall B-factor refinement. As in the AMP-PCP bound structure, density for the far C-terminus was not well defined. A weakly bound ligand, tentatively assigned as chloride ion, was identified in the nucleotide binding site of subunits A and C by inspection of the 2F_o-F_c and F_o-F_c electron density maps.

Evaluation

Buried surface areas were calculated using the web-based program PISA⁴² and analyzed using the Protein Interaction Calculator⁴³. The AMP-PCP bound PilT, subunit B, structure was processed using the web-based version of ConSurf¹⁶. The PilT sequence was aligned using default parameters with other secretion NTPase protein sequences (26 sequences in total) with an E value cut off of 0.001.

Structure figures were made with PyMOL⁴⁴.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1. The structure of AMP-PCP bound PilT

a) AMP-PCP (yellow carbons, blue nitrogens, red oxygens, orange phosphates) is bound at the interface of the two domains, surrounded by conserved motifs of the secretion ATPases (Walker A, $\alpha 6$, red; Walker B, $\beta 10$, blue; Asp Box, $\beta 8$, yellow; His Box, $\beta 11$, orange; all shown on subunit B). AMP-PCP is distant from the AIRNLIRE helix required for retraction (cyan).

b) The PilT hexamer, looking down the crystallographic 2-fold axis with the N-terminal domains oriented towards the reader (subunit A, green; B, blue; C, purple; the second subunit C is grey to match the subunit shown in panel a; ligands colored as in panel a).

FIGURE 2. Conservation of PilT residues among secretion ATPases

Among 27 secretion ATPases, as mapped onto PilT, the most conserved residues (reddish-brown) are in the active site cleft or in proximity to the active site cleft of the neighboring subunit (grey ribbons). The least conserved residues (deep teal) are located on the periphery of the subunits. The active site ligands are colored as in Figure 1.

FIGURE 3. The nucleotide binding site and the His Box of PiIT

a) Wall-eyed stereo view of the active sites of liganded (blue subunit B, green subunit A, grey Mg^{2+} , red water) and apo (light blue subunit B, light green subunit A) PiIT, aligned using the conserved RecA fold of the CTD (residues 106–301). In addition to residues discussed in the text, Arg276 coordinates the ribose while Leu109 and Leu268 sandwich the adenine moiety of the AMP-PCP. The $F_o - F_c$ omit map was calculated from the ligand bound structure without AMP-PCP or Mg^{2+} ion and is contoured at 3σ .

b) Thr220 and His222 (green, subunit A) and Thr132 and His229 (blue, subunit B) form a 3D His Box in the crystal structure.

FIGURE 4. Ready, Active, Release conformations of the clamp arginines of the 3 PilT subunits
 a) Arginines 82 and 97 of subunit A are in a **Ready** conformation to bind the nucleotide. (Colors as in Fig. 1b and 3). b) The clamp arginines of subunit B are in an **Active** conformation, coordinating the γ -phosphate of the nucleotide. c) The clamp arginines have **Released** their hold on the nucleotide. d) A cartoon schematic representing the orientation of the NTDs with respect to the superimposed RecA CTDs. (Green, subunit A; blue, subunit B; purple, subunit C.)

FIGURE 5. Force generation by large domain motions among hexameric ATPase proteins ATP binding leads to large domain movements (red arrows) within the subunit (shown in light blue cartoon). Left panels show two separate hexamer views with each subunit individually colored. The zoom in is of a single subunit (light blue) illustrating the motions of the moving domain during ATP binding, with the RecA domains fixed. Secretion ATPases (a) PilT (Chain A apo and Chain C – AMP-PCP), (b) GspE (2OAP, 2OAQ) and (c) HP0525 (1NLV, 1NLZ) exhibit domain motions that are diagonal to the central ring axis, while (d) HslU (1G3I, 1DO2) and (e) F₁-ATPase (1BMF) have domain motions which are parallel and perpendicular to the ring axis, respectively.

FIGURE 6. Schematic model for pilus retraction

(A) If PilT were to act on pilin directly, the NTD of PilT (blue) would contact the N-terminal tail of the bottom-most pilin subunit (red, modeled with a PilT-induced kink at proline 22) in a Type IV pilus filament (2HIL²⁷) undergoing disassembly across the inner membrane and guided through the outer membrane by the PilQ secretin (brown). (B) More likely, inner membrane proteins are also involved in the pilus retraction pathway, and the force generated upon domain closure by PilT is transferred through an inner membrane protein to the pilin subunits, thereby providing the energy needed to disrupt hydrophobic and polar pilin:pilin interactions. The association of PilT with any particular inner membrane protein remains an unproven hypothesis. Shown are one pilus filament, one PilM, a heterodimer of PilN:PilO⁶ and one PilC (for simplicity; PilC is likely a dimer⁴⁵). Based on membrane topology predictions⁴⁶ for the 406 residue *P. aeruginosa* strain PA01 inner membrane protein PilC, we have represented PilC with three full transmembrane helices and two stubby membrane-embedded reentrant helices. This prediction should be viewed with caution, as other publicly available topology analysis algorithms⁴⁷ yield varying results.

Table 1

Data collection and refinement statistics

Data Collection	AMP-PCP bound PilT	Unliganded PilT
Wavelength (Å)	0.900	0.979
Space group	C222 ₁	C222 ₁
a, b, c (Å)	108.5, 119.6, 185.5	108.2, 121.4, 184.4
Resolution (Å)	30.0–2.60 (2.69–2.6)	30.00–3.10 (3.21–3.1)
Unique reflections (#)	35387 (3452)	21079 (1652)
R _{merge}	0.052 (0.422)	0.098 (0.384)
I/σ ₁	32.6 (2.8)	17.3 (3.1)
Completeness (%)	94.4 (93.6)	94.9 (76.1)
Redundancy	7.4 (4.1)	7.0 (5.6)
Wilson B-factor (Å ²)	68.8	59.8
Refinement		
Resolution	29.1–2.60 (2.66–2.60)	23.3–3.10 (3.26–3.10)
Reflections	33594 (2319)	20121 (2376)
R _{work} /R _{free}	0.244/0.291 (0.352/0.416)	0.229/0.282 (0.331/0.442)
No. atoms	7939	7643
Protein	7741	7686
Ligand/ion	109	15
Water	89	4
B-factor (Å ²)	61.0	63.6
AMP-PCP (A/B/C)	70.4/60.9/70.7	n/a
RMS deviations		
Bond lengths (Å)	0.010	0.014
Bond angles (°)	1.434	1.477
Ramachandran		
Most favored	95.8%	86.3%
Outliers	0.41%	0.82%
ESU (maximum likelihood)	0.30 Å	0.42 Å

* Parentheses indicate highest resolution shell.