The ATPase Activity of BfpD Is Greatly Enhanced by Zinc and Allosteric Interactions with Other Bfp Proteins*

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Type IV pilus biogenesis, protein secretion, DNA transfer, and filamentous phage morphogenesis systems are thought to possess similar architectures and mechanisms. These multiprotein complexes include members of the PulE superfamily of putative NTPases that have extensive sequence similarity and probably similar functions as the energizers of macromolecular transport. We purified the PulE homologue BfpD of the enteropathogenic Escherichia coli bundle-forming pilus (BFP) biogenesis machine and characterized its ATPase activity, providing new insights into its mode of action. Numerous techniques revealed that BfpD forms hexamers in the presence of nucleotide. Hexameric BfpD displayed weak ATPase activity. We previously demonstrated that the N termini of membrane proteins BfpC and BfpE recruit BfpD to the cytoplasmic membrane. Here, we identified two BfpD-binding sites, BfpE39–76 and BfpE77–114, in the N terminus of BfpE using a yeast two-hybrid system. Isothermal titration calorimetry and protease sensitivity assays showed that BfpD-ATP·S binds to BfpE39–76 and BfpD-ADP binds to BfpE77–114. The ATPase activity of hexameric BfpD was enhanced up to 1200-fold to a maximum of 75.3 μmol of Pi min⁻¹ mg⁻¹ protein. Zinc ions were required for BFP biogenesis and function (4, 19–26). The bfpD gene encodes a member of the PulE superfamily of putative nucleotide-binding proteins involved in bacterial type II and IV secretion, Tfp assembly, natural competence, and assembly of archaean flagellae (17). These proteins contain several conserved

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sequence motifs, including Walker A and B boxes involved in ATP binding and hydrolysis (30). However, ATPase activity has been confirmed for only a few of them, mostly from type IV secretion systems (31–38). Furthermore, this ATPase activity has been relatively weak, between 0.71 and 61 nmol of inorganic phosphate (P_i) released min^-1 mg^-1 of protein. Mutation of key residues in the Walker A box of PuLE superfamily members abolishes activity of the cognate systems, for example, PuLE (39, 40), EpsE (41), OutE (42), PilB (43), and BfpD (4). Therefore, these proteins most likely share the function of supplying energy from ATP hydrolysis to drive organelar assembly or substrate translocation.

The characterization of the ATPase activity of BfpD is crucial for understanding its role in BFP biogenesis and mechanism of action. In this paper we describe a detailed study of the ATPase activity of BfpD in which we address its activity in the context of the BFP biogenesis machine, i.e., in the presence of components of the machinry with which it interacts. The results of our studies suggest a model for conversion of chemical energy into mechanical energy to power extraction of bundlin from the cytoplasmic membrane for assembly into BFP filaments.

**MATERIALS AND METHODS**

**Protein Purification**

His-tagged BfpD and the cytoplasmic N terminus of BfpC (amino acids 1–164) were purified from *E. coli* strains BL21/D3/Iys/Lsp/RPA405 and DH5/pRPA302, respectively, as described previously (29). Briefly, DH5/pRPA302 and BL21/D3/Iys/Lsp/RPA405 cultures were grown at 37 °C to an optical density at 600 nm of ~0.5. His-tagged protein was induced with 1 mM isopropyl 1-thio-galactopyranoside (IPTG) and was grown for an additional 2 and 5 h, respectively. Cells were then harvested by centrifugation for 20 min at 4,000 × g and lysed in a buffer containing 50 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0, twice (10,000 × g, 10 min) and BfpC and BfpE, and 20 °C for 5 min. Data were analyzed with Dynals software.

**ATPase Activity of BfpD**

Dynamic light scattering (DLS) measurements were carried out using a DynaPro 99 photometer (Protein Solutions). BfpD (0.2–0.6 mg ml^-1) in 20 mM Tris-HCl buffer, pH 7.5, 100 mM NaCl, 10 mM MgCl_2, containing 1 mM ATP, 1 mM ATP/S, or 1 mM ADP was measured at 20 °C for 5 min. Data were analyzed with Dynals software.

**Electron Microscopy**

Negative staining—Carbon-coated copper grids were floated on 5-μl drops of 100 mg ml^-1 BfpD in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl_2, 1 mM ATP/S. After 5 min the sample was wicked off, and grids were floated consecutively on 3 drops (60 μl) of 3% uranyl acetate, with blotting in between. Grids were floated on the third drop for 1 min, then blotted and air-dried. Images were recorded on a Philips/FEI CM100 electron microscope operating at 100 kV in low dose mode at ×52,000 magnification and a defocus of 500 nm.

Cryo-electron microscopy—Five-μl drops of BfpD were applied to Quantifoil holey grids (Quantifoil Micro Tools GmbH) that had been glow-discharged in the presence of amyl amine. After 1 min the grids were blotted for 2.5 s and then plunged into an ethane slush cooled with liquid nitrogen, using the Vitrobot (FEI). Grids were transferred to a Gatan 626 cold stage (Gatan), and images were recorded on a Philips/FEI CM120 electron microscope operating at 120 kV in low dose mode at ×50,000 magnification and a defocus of 1 μm.

**Yeast Two-hybrid Experiments**

We used the Matchmaker GAL4 two-hybrid system 3 (Clontech) following the protocol suggested by the manufacturer. The cloning of DNA fragments encoding BfpD and the N terminus of BfpE into pGBKT7 (GAL4 DNA-binding domain vector) and pGADT7 (GAL4 activation domain vector) has been described previously (29). In addition, PCR-amplified fragments encoding amino acids 1–38, 39–76, 77–114, 1–76, and 39–114 of the N terminus of BfpE (for primers see Table I) were cloned into these vectors. *Saccharomyces cerevisiae* strain AH109 was co-transformed with these constructs by the lithium acetate procedure. The transformants were plated on medium containing X-gal and lacking tryptophan, leucine, histidine, and adenine and assayed for α-galactosidase activity to detect and quantify interactions.

**Isothermal Titration Calorimetry**

Isothermal titration calorimetry (ITC) experiments were carried out using a VP-ITC Microcalorimeter (Microcal). Peptides of BfpE_39–76

**Zonal Sedimentation Analysis**

Purified BfpD (20 μg) was applied to the top of a 2.5 ml 20–40% sucrose gradient formed in 20 ml Tris-HCl buffer, pH 7.6, 100 mM NaCl, 10 mM MgCl_2, with 1 mM ATP as appropriate. Gradients were centrifuged at 245,000 × g for 12 h at 4 °C. Fractions (100 μl) were collected, precipitated with trichloroacetic acid, and subjected to SDS-PAGE and immunoblotting. Molecular mass standards run in parallel gradients were visualized by SDS-PAGE and Coomassie staining. Sedimentation coefficient (s_20,w) values were determined by sedimentation relative to protein standards.

Molecular mass values were calculated from the equation molecular mass = 6N_M w/v_p, where N is Avogadro's number, a is the Stokes radius, _n_ is the viscosity of water at 20 °C, _v_ is the partial specific volume of the protein calculated from its amino acid composition, and _p_ is the density of water at 20 °C (as described (45)).
**Fluorescence Measurements**

The fluorescent nucleotide analogues 2′-O-(4-aminophenyl)-ATP and TNP-ADP were used to study nucleotide binding. Experiments were performed at 37 °C with various concentrations of nucleotide in 20 mM Tris-HCl buffer, pH 7.6, containing 100 mM NaCl and 10 mM MgCl₂. The fluorescence in the presence of BfpD, proportional to the quantity of BfpD-bound TNP-nucleotide, was plotted as a function of TNP-nucleotide concentration. Data were fitted to the equation

\[
\frac{F}{\Delta F} = \frac{[S]}{K_{d} + [S]},
\]

where \(\Delta F\) is the change in fluorescence intensity of TNP-nucleotide, \([S]\) is the nucleotide concentration, and \(K_{d}\) is the dissociation constant. The stoichiometry of BfpD's interaction with TNP-nucleotide was derived using the method of Stinson and Holbrook (47). Briefly, 1/(1/[S]) was plotted against \([S]/\alpha\), where \(\alpha\) is the fractional occupation of the TNP-nucleotide-binding sites (\(\Delta F/\Delta F_{\text{max}}\)). A straight line with a slope of \(1/K_{d}\) and an \(x\)-intercept of the concentration of TNP-nucleotide-binding sites was obtained. Stoichiometry of binding was then calculated from the amino acid sequence or estimated, but not its hydrolysis, is required for BfpD hexamerization. To determine the molecular masses of the two BfpD forms, parameters obtained from gel filtration (Stokes radii) and zonal sedimentation (sedimentation coefficients) were analyzed (Table II). These indicate that the BfpD in the slower peaks was monomeric BfpD (molecular mass = 60 kDa), whereas that in the faster peaks was hexameric BfpD (molecular mass = 369 kDa). In addition, DLS analysis of the high molecular mass form of BfpD in the presence of ATP, ATP·γS, or ADP showed that BfpD is a hexamer (Table III).

Electron microscopy studies revealed that hexameric BfpD had a ring-like structure (Fig. 1, B–E). The electron micrograph of negatively stained BfpD in Fig. 1B shows ring-like structures in which subunits are evident. The diameters of these rings measure ~11.5 nm. Furthermore, the rings in the electron micrographs of frozen-hydrated BfpD (Fig. 1, C–E) display 6-fold symmetry, suggesting a hexamer structure consistent with the gel filtration, zonal sedimentation, and DLS results. Taken together, these findings demonstrate that BfpD assembles to form hexamers in the presence of ATP, ATP·γS, TNP-ATP, ADP, and TNP-ADP.

**Proteinase K Digestion**

All protease digestions were carried out at room temperature in 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 10 mM MgCl₂, containing 1 mM ATP·S or 1 mM ADP as appropriate. Hexameric BfpD (5 μg), purified in the presence of ATP·S or ADP, was preincubated with BfpE₇₋₇₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋˓→

**RESULTS**

*BfpD Is a Hexamer*—BfpD synthesized with a N-terminal His tag in *E. coli* strain BL21pLysS,pRPA405 was purified to homogeneity using two chromatographic steps (for BfpD purity, see Fig. 6). BL21pLysS,pRPA405 produces functional BfpD as plasmid pRPA405, encoding the His-tagged BfpD, complements the *bfpD* mutant UMD926 to restore autoaggregation, a phenotype that correlates with biogenesis of functional BFP (results not shown). The elution profile of BfpD during Sephacryl S-300 gel filtration chromatography, the second purification step, was dependent on ATP and MgCl₂ (Fig. 1A). In the absence of both ATP and MgCl₂ and in the absence of ATP but the presence of MgCl₂, BfpD eluted as a sharp peak with an estimated molecular mass of 60 kDa (see below). In contrast, the molecular mass of BfpD obtained when MgCl₂ was present in the amino acid sequence or estimated, but not its hydrolysis, is required for BfpD hexamerization. To determine the molecular masses of the two BfpD forms, parameters obtained from gel filtration (Stokes radii) and zonal sedimentation (sedimentation coefficients) were analyzed (Table II). These indicate that the BfpD in the slower peaks was monomeric BfpD (molecular mass = 60 kDa), whereas that in the faster peaks was hexameric BfpD (molecular mass = 369 kDa). In addition, DLS analysis of the high molecular mass form of BfpD in the presence of ATP, ATP·γS, or ADP showed that BfpD is a hexamer (Table III).

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**Hexameric BfpD Is an ATPase**—We detected the ATPase activity of BfpD using a colorimetric malachite green assay to measure the amount of Pi released upon incubation with ATP. BfpD purified in 40 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl and 1 mM ADP was used for these ATPase assays. Use of ADP instead of ATP in the BfpD purification produces hexameric BfpD and has the advantage of eliminating high background P₄ levels. ADP does not inhibit BfpD ATPase ac-
The optimal concentration of NaCl was found to be 150 mM occurred at pH 7.0 (data not shown). In additional experiments, activity was strongly inhibited in the presence of divalent cations (Fig. 2A) and activity was strongly inhibited in the presence of the divalent cation chelator EDTA (data not shown). Interestingly, hexameric BfpD exhibited the highest activity in the presence of Zn2+ (Fig. 2A). Substitution of Zn2+ by Mg2+ and Mn2+ decreased the ATP hydrolizing activity to 90 and 84%, respectively, of that obtained with Zn2+, whereas substitution by Ca2+, Cu2+, Fe2+, Co2+, Cd2+, and Ni2+ reduced it to less than 10%. Therefore, ATPase assays were performed in 40 mM Tris-HCl, pH 7.0, 150 mM NaCl, 10 mM ZnCl2, and 5% glycerol.

When hexameric BfpD protein was incubated with ATP, release of P_i occurred in a time- and BfpD concentration-dependent manner (Fig. 2, B and C), indicating that hexameric BfpD has ATPase activity. That this activity was because of BfpD rather than a contaminating ATPase is demonstrated by the fact that preparations obtained by an identical purification scheme from cells containing plasmid lacking bfpD did not display ATPase activity. Interestingly, monomeric BfpD only began to display ATPase activity after 30 min (data not shown). Using gel filtration chromatography, we found that monomeric BfpD hexamerizes in the presence of nucleotide, such as the ATP added to initiate ATP hydrolysis (data not shown). Therefore, we believe that the ATPase activity exhibited by monomeric BfpD was actually that of hexacemous BfpD. Finally, BfpD did not hydrolyze any other nucleotides (ADP, AMP, GTP, CTP, and UTP) tested in substrate specificity experiments (data not shown).

**Table II**

| Values represent the mean ± S.E. of at least 3 experiments. | Gel elution peak | Stokes radius nm | Sedimentation coefficient S kDa | Monarch coefficient a | Molecular mass kDa |
|----------------|-----------------|-----------------|-------------------------------|----------------------|-------------------|
| Slower         | 4.6             | 0.8             | 7.40                          | 0.98                 | 364               |
| Faster         | 7.6             | 1.2             | 11.1                          | 1.2                  | 369               |

**DLS measurements for BfpD**

| Nucleotide | R_ha | R_hb |
|------------|------|------|
| ATP        | 7.40 | 7.40 |
| ATPγS      | 7.40 | 7.40 |
| ADP        | 7.45 | 7.45 |

a Calculated hydrodynamic radius.

b Standard deviation of the Gaussian model.
These interactions occurred irrespective of which protein was fused to the activation and which to the DNA-binding domains of GAL4. The remaining transformants did not grow on medium containing X-/H9251-Gal and lacking tryptophan, leucine, histidine, and adenine and exhibited low levels of /H9251-galactosidase activity, similar to the negative control expressing human lamin C and SV40 large T-antigen fusion proteins (Fig. 3). Western analysis showed that the transformants expressed the Bfp fusion proteins at comparable levels (results not shown). Furthermore, S. cerevisiae AH109 cells co-transformed with each construct individually and either "empty" pGBK7 or pGADT7 grew on the appropriate selective medium, i.e. they did not grow on medium containing X-α-Gal and lacking tryptophan, leucine, histidine, and adenine and

### FIG. 2. ATPase activity of BfpD.

ATPase assays were carried out at 37 °C in 40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5% glycerol, 5 mM ATP, and either 10 μM Mg, Mn, Ca, Cu, Zn, Fe, Co, Cd, or Ni. The (A) and (D) dependence of the ATPase activity on divalent cation concentration is shown. The ATP concentration dependence of the ATPase activity is shown in (B) and (C), and ATP concentration was 0, 0.1, 0.5, 1, 2, 5, 7.5, 10, and 100 mM. The ATPase activity is displayed as activity (μmol min⁻¹ mg⁻¹) at each concentration of divalent cation. The data were fitted with a one-site model to give the association constant (K), stoichiometry (molar ratio of BfpD to peptide, n), and enthalpy change (ΔH) upon peptide-BfpD complex formation.

### TABLE IV

| Parameter | BfpD | BfpD + BfpC | BfpD + BfpE77–114 |
|-----------|------|------------|-------------------|
| Vₘₐₓ     | 0.06 ± 0.0 | 58.70 ± 2.2 | 75.30 ± 2.3 |
| Kₘ     | 24.0 ± 0.7 | 6.0 ± 0.2 | 5.9 ± 0.2 |

### Differential Nucleotide-dependent BfpD Affinity for BfpE₉₉–₇₆ and BfpE₇₇–₁₁₄

We speculated that BfpD preferentially binds to each of the BfpD-binding sites of the N terminus of BfpC at different stages of catalysis of ATP hydrolysis. To test this hypothesis and to confirm interactions between BfpD and BfpE₉₉–₇₆ and BfpE₇₇–₁₁₄, the thermodynamics of the interactions were measured by ITC. For these purposes we synthesized peptides corresponding to BfpE₉₉–₇₆ and BfpE₇₇–₁₁₄. Fig. 4 shows calorimetry data for titrations of these peptides with hexameric BfpD in the presence of ATPγS (a non-hydrolysable ATP analogue) and ADP. Large exothermic enthalpies were observed in titrations of BfpD-ATPγS into BfpE₉₉–₇₆ and BfpD-ADP into BfpE₇₇–₁₁₄. These data do not distinguish whether these sites represent two independent BfpD-binding sites or whether these sites cooperate to form one binding site.
formation (49). These parameters show that one molecule of hexameric BfpD strongly interacts with six molecules of BfpE/peptide (K = 1.33 × 10^7 ± 2.6 × 10^6 M⁻¹, K_d = 75 nm, n = 0.162 ± 2.62 × 10⁻⁴, ΔH = -1.02 × 10⁶ ± 242 cal/mol) and that one molecule of hexameric BfpD-ATP₇₆ strongly interacts with three molecules of BfpE/peptide (K = 1.39 × 10⁶ ± 2.04 × 10⁵ M⁻¹, K_d = 72 nm, n = 0.334 ± 3.88 × 10⁻⁴, ΔH = -3.36 × 10⁵ ± 577 cal/mol). Importantly, hexameric BfpD-ATP₇₆ showed no measurable interaction with BfpE/peptide, whereas those that did not grow are shown in white. We think that this interaction is likely to be biologically relevant.

Hexameric BfpD Binds Six Molecules of the Fluorescent Nucleotide Derivatives TNP-ATP and TNP-ADP—To determine whether the stoichiometry of binding of BfpD to BfpE/peptide reflects the stoichiometry of binding of BfpD to ATP₇₆ and ADP, respectively, we used the non-hydrolysable nucleotide analogues TNP-ATP and TNP-ADP to ascertain the parameters of BfpD binding to nucleotides. TNP-nucleotides are weakly fluorescent in aqueous solution, however, their fluorescence is greatly enhanced in a hydrophobic environment such as the nucleotide-binding site of a protein. Upon excitation at 408 nm, each TNP-nucleotide in solution exhibited a characteristic fluorescence emission with a maximum at 535 nm. Non-hydrolysable nucleotide BfpD markedly increased the fluorescence and blue shifted the maximum to 535 nm. Thus, monomeric BfpD was incubated with TNP-ATP and TNP-ADP and the increase in the fluorescence signal compared to the negative control as described by the equation:

\[
\text{Fluorescence (Stoichiometry)} = \text{Fluorescence (BfpD + TNP-nucleotide)} - \text{Fluorescence (BfpD)}
\]

When the fluorescence was measured using a standard microplate reader, the stoichiometry of binding of BfpD to ATP and ADP was 1.07 for TNP-ATP and 0.98 for TNP-ADP. These results indicate that one molecule of TNP-ATP or TNP-ADP binds to one molecule of either TNP-nucleotide. Because monomeric BfpD hexamerizes in the presence of either TNP-ATP or TNP-ADP, we can assume that one molecule of hexameric BfpD binds six molecules of TNP-nucleotides, each BfpD monomer of the hexamer binding one TNP-nucleotide molecule. Thus, the inability of hexameric BfpD to bind to more than three TNP-nucleotide molecules is not because of an inability of each subunit to bind ATP.
BfpE Binding Increases BfpD Susceptibility to Protease K Digestion—We have previously shown that BfpD and the N terminus of BfpC induce reciprocal conformational changes upon binding to one another (29). We hypothesized that a BfpD subunit of hexameric BfpD-ADP binds BfpE[39–76], whereas alternate BfpD subunits of BfpD-ATP/S bind one molecule of BfpE[39–76] and BfpE[77–114] upon binding to one another (29). We hypothesized that each of these cases, it suggests that binding of BfpD-ADP and BfpD-ATP/S to BfpE[39–76] and BfpE[77–114] induces conformational changes that greatly enhance the proteinase K sensitivity of BfpD. We showed in Fig. 5 that BfpE[39–76] and BfpE[77–114] significantly increased the sensitivity of BfpD-ATP/S to digestion by proteinase K. In contrast, BfpE[77–114] significantly increased the sensitivity of BfpD-ATP/S to digestion by proteinase K. In addition, BfpE[77–114] modestly but reproducibly increased the sensitivity of BfpD-ATP/S to proteolysis. These results show that BfpE[77–114] only had a detectable effect on the proteinase K sensitivity of the form of BfpD with which our ITC data demonstrates they interact. Furthermore, they suggest that BfpE[39–76] and BfpE[77–114] induce changes in the conformation of BfpD-ADP and BfpD-ATP/S, respectively, upon binding, which account for the observed increases in sensitivity to proteinase K digestion. However, consistent with our hypothesis, BfpD-ADP and BfpD-ATP/S undergo different conformational changes as they display differing proteinase K sensitivities.

BfpD Binding to the N Terminus of BfpC and BfpE[77–114] Dramatically Increases Its ATPase Activity—Because hexameric BfpD interacts with the N terminus of BfpC, BfpE[39–76] and BfpE[77–114] (Ref. 29, and this study), we investigated the influence of BfpE on the ATPase activity of BfpD. Hexameric BfpD, alone and together with BfpE[39–76] or BfpE[77–114] in the presence of ATP/S (top panel) or ADP (bottom panel), was incubated with the indicated concentrations of proteinase K. Digestion was monitored by enhancement of fluorescence.

**Figure 5.** Binding of TNP derivatives of ATP and ADP to BfpD as monitored by enhancement of fluorescence. Monomeric BfpD was incubated with various concentrations of TNP-ATP (○) and TNP-ADP (●) and the fluorescence enhancement (∆F) was measured (excitation 408 nm, emission 535 nm). Binding curves were fitted to an equation describing binding to a single affinity site (solid lines), and values for the dissociation constant and the maximum fluorescence enhancement (∆Fmax) were extracted. Results of three independent titrations are included.

**Figure 6.** BfpD-ATP/S and BfpD-ADP binding to BfpE[77–114] and BfpE[39–76] respectively, enhances the proteinase K sensitivity of BfpD. Hexameric BfpD, alone and together with BfpE[39–76] or BfpE[77–114] in the presence of ATP/S (top panel) or ADP (bottom panel), was incubated with the indicated concentrations of proteinase K. Proteolytic digests were subjected to SDS-PAGE followed by silver staining.

DISCUSSION

Tfp biogenesis, protein secretion, DNA transfer, archaellar flagellar assembly, and filamentous phage morphogenesis systems are multiprotein assemblies constructed in part from...
proteins conserved between the systems composing these macromolecular transport systems, putative nucleotide-binding proteins of the PulE superfamily are widely conserved between these systems. Of the proteins previously shown that BfpD interacts with the cytoplasmic N termini of BfpC and BfpE1–114 and is recruited to the cytoplasmic membrane by both BfpC and BfpE (29). Here we further define the BfpC and BfpE sites of BfpD and show that BfpD binds to BfpC and BfpE depending on the stage of the catalytic cycle. The form of BfpD binds with high affinity (Kd ~ 75 nM) to BfpE39–76, and the ADP-bound form of BfpD interacts with the N terminus of BfpC and BfpE77–114 together more than 1200-fold to a Vmax of 0.71 to 61 nmol of Pi released min−1 mg−1 of protein. However, the ATPase activity in the presence of BfpE77–114 had no detectable effect on BfpD ATPase activity, a result that is not surprising, as BfpD-ATP does not interact with BfpE39–76. The allosteric effects of BfpC and BfpE on BfpD ATPase activity indicate that BfpC and BfpE indirectly play a major role in supplying the BFP biogenesis machine with the energy it requires for its assembly, bundlin polymerization, and/or transmembrane transport. Not only do they recruit BfpD to the biogenesis machinery, they also dramatically increase its ATPase activity. In this way, the ATPase activity of BfpD is regulated to be maximal at the site where energy is needed for BFP formation, making the process of producing BFP energy efficient and minimizing wasted energy expenditure by the free cytoplasmic enzyme. Interestingly, monomeric BfpD preparations exhibited delayed ATPase activity that was greatly increased by the N terminus of BfpC and BfpE77–114, but not BfpE39–76. This ATPase activity was most likely that of BfpD hexamers formed in the presence of the ATP added in the assay, because monomeric BfpD does not interact with the N terminus of BfpC, BfpE39–76, or BfpE77–114. Our data suggest that nucleotide binding induces formation of BfpD hexamers, which are selectively recognized, recruited, and stimulated by membrane-bound BfpC and BfpE, thus maximizing the energy supplied to the BFP biogenesis machine.

We found that hexameric BfpD binds to a maximum of three molecules of BfpE77–114 in the presence of ATP, but can bind to other PulE superfamily members (33, 34, 37, 52–54) and seems to be a common characteristic of members of this superfamily. These hexameric ring assemblies are reminiscent of those formed by nucleotide-dependent molecular motors such as helicases and F1-ATPases (52, 54, 55), and membrane fusion ATPases such as N-ethylmaleimide-sensitive fusion protein and p97 (56, 57).

The ATPase activity measured for purified hexameric BfpD (Vmax of 62.2 nmol of Pi released min−1 mg−1 of protein) is similar in magnitude to the highest values previously measured for other members of the PulE superfamily (ranging from 0.71 to 61 nmol of Pi released min−1 mg−1 of protein). We considered the weak ATPase activities of these PulE superfamily members to be because of the absence of stimulatory proteins of the cognate systems and/or the reaction conditions used. After optimizing our reaction conditions, we tested whether components of the BFP biogenesis apparatus that interact with BfpD stimulate its ATPase activity. We have previously shown that BfpD interacts with the cytoplasmic N termini of BfpC and BfpE (BfpE39–76 and BfpE77–114) unless otherwise indicated (see “Experimental Procedures”). In combination of BfpC, BfpE39–76, and BfpE77–114, assays were performed in the presence of the indicated nucleotide (ATP, ATPS, TNP-ATP, ADP, and TNP-ADP). A combination of gel filtration, zonal sedimentation, DLS, and electron microscopy confirmed that BfpD forms ring-shaped hexamers in the presence of nucleotide (ATP, ATPγS, and ADP). The ability to form hexameric rings has been reported for other PulE superfamily members (33, 34, 37, 52–54) and seems to be a common characteristic of members of this superfamily. These hexameric ring assemblies are reminiscent of those formed by nucleotide-dependent molecular motors such as helicases and F1-ATPases (52, 54, 55), and membrane fusion ATPases such as N-ethylmaleimide-sensitive fusion protein and p97 (56, 57).

We purified BfpD using an approach including Sephacryl S-300 gel filtration chromatography and observed that BfpD eluted as a hexamer as well as a monomer in the presence of nucleotide (ATP, ATPγS, TNP-ATP, ADP, and TNP-ADP). A combination of gel filtration, zonal sedimentation, DLS, and electron microscopy confirmed that BfpD forms ring-shaped hexamers in the presence of nucleotide (ATP, ATPγS, and ADP). The ability to form hexameric rings has been reported
six molecules of BfpE_{39–76} in the presence of ADP. This difference in stoichiometry is not because of differences in nucleotide saturation, as BfpD is able to bind six molecules of either ATP or ADP. Rather these data suggest that occupancy of the ATP-activated BfpD hexamers by BfpE occurs at every other subunit and induces a conformational change that precludes binding of adjacent subunits.

Hexameric BfpD exhibited the highest ATPase activity in the presence of Zn^{2+} and the dramatic stimulation of its activity by the N terminus of BfpC and BfpE_{77–114} occurred only in the presence of Zn^{2+}. The crystal structure of a truncated form of the closely related type II secretion ATPase EpsE (missing ninety N-terminal amino acids) of V. cholerae has been solved, revealing a molecule with four domains, N2, C1, CM, and C2, and inferring a missing N1 domain. Electron density most consistent with the presence of a Zn^{2+} ion was detected in the CM domain, coordinated by four cysteines. This location is remote from the active site. An alignment of BfpD and EpsE reveals that these cysteine residues are conserved. It is not clear therefore, whether Zn^{2+} is catalytically relevant or merely structurally relevant for either EpsE or BfpD. For example, Zn^{2+} binding might be necessary for conformational changes that allow binding of BfpE or BfpC. However, this hypothesis is not supported by our ITC data, which were acquired in the presence of Mg^{2+}, not Zn^{2+}. Therefore, we propose that the Zn^{2+} ion might perform a critical catalytic role for BfpD and perhaps for other members of the PulE ATPase family. In our assays, we purified hexameric BfpD in buffer lacking divalent metal ions and measured ATPase activity in the presence of buffer containing various divalent ions. We cannot be certain that trace quantities of metal were not present in any of the buffers used. BfpD purifed inductively coupled plasma optical emission spectroscopy that an equimolar ratio of Zn^{2+} was present. Rather these data suggest that occupancy of the ATP-binding site is required in the presence of Zn^{2+}.

The crystal structure of a truncated form of BfpD was solved and reveals that the N terminus of BfpE is involved in interactions with other essential proteins that are necessary for BFP biogenesis (29); (iii) hexameric BfpD exhibits high ATPase activity in the presence of other essential components of the BFP biogenesis machine, consistent with the idea that it is a molecular motor; and (iv) hexameric BfpD displays differential binding to two sites of the N terminus of BfpE depending on the phospho-

![Image](http://example.com/image.png)

**FIG. 8.** Schematic diagram of the proposed mechanism by which BfpD transduces chemical energy to the BFP biogenesis machine. ATP binds to the N terminus of BfpC and to the BfpE and BfpE_{77–114} octamers. This greatly increases the ATPase activity of BfpD. Therefore, the ATP binding drives the movement of the N terminus of BfpE from the Cytoplasm to the Periplasm, allowing BfpD to interact with the periplasmic membrane. This movement drives the extraction of bundlin from the cytoplasmic membrane and the dramatic stimulation of its activity by the N terminus of BfpC and BfpE_{77–114}. The figure is simplified to show the movement of only one monomer, but presumably occurs simultaneously at alternating monomers. Hexameric BfpD binds to the cytoplasmic N terminus of BfpC and BfpE_{77–114}, which also interact with each other. BfpD-ATP binds to BfpE_{77–114}, the domain of the N terminus of BfpE closest to the cytoplasmic membrane. Conformational changes in BfpD upon binding to the N terminus of BfpC and BfpE_{77–114} greatly increase its ATPase activity. Subsequently, the BfpD-ADP resulting from ATP hydrolysis shifts to BfpE_{39–76} while remaining bound to the N terminus of BfpC. It seems unlikely that BfpD moves to its new binding site in BfpE as its movement is restricted by its interaction with the N terminus of BfpC. Therefore we propose that the N terminus of BfpE is raised allowing BfpD-ADP to interact with BfpE_{39–76}. This movement may force the most distal part of the N terminus of BfpE into the cytoplasmic membrane and perhaps into the periplasm. The interaction between the N terminus of BfpC and BfpE_{39–76} presumably limits the distance that the N terminus of BfpE is pushed through the cytoplasmic membrane. According to this model, the BfpD motor energizes BfpE to act as a piston, whereas BfpC serves as a scaffold protein, anchoring BfpD and the N terminus of BfpE in place. Preliminary studies indicate that other essential Bfp proteins bind to BfpE_{77–114}, which could be transported across the cytoplasmic membrane in association with the N terminus of BfpE. In this way, BfpD provides the necessary mechanical force for recruitment and assembly of Bfp proteins into the BFP biogenesis machine.

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*L. J. Crowther, A. Daniel, and M. S. Donnenberg, unpublished results.*
machine, which results in the extraction of nascent bundlin from the cytoplasmic membrane and incorporation into the growing pilus filament. Finally, BfpD is replenished with ATP and released from BfpP39–76 and the cytoplasmic membrane subassembly of the BFP biogenesis machine relaxes, allowing the distal part of the N terminus of BfpE to slip back through the cytoplasmic membrane. Whereas further experiments are necessary to test this model of energy transduction during BFP biogenesis, the insights into the function and mode of action of BfpD we report here increase our understanding of the mechanism of Tfp biogenesis machines and have important implications for similar systems that transport biological macromolecules across membranes. We propose that this model is relevant to the entire PulE superfamily. Orthologues of BfpE (GspF) are highly conserved in systems that include PulE superfamily members (17). Although BfpC orthologues are not common, GspL and GspM proteins, which span the cytoplasmic membrane, interact with each other and recruit PulE family ATPases to the cytoplasmic membrane (41, 60), are widespread and likely play roles similar to that carried out by BfpC. Furthermore, the unique properties of BfpD suggest that drug therapy targeting the ATPase activity of PulE superfamily proteins and their interactions with proteins of their cognate systems could be designed to disrupt these macromolecular machines and the virulence properties they control.

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