Construction of Asymmetrical Hexameric Biomimetic Motors with Continuous Single-Directional Motion by Sequential Coordination

Zhengyi Zhao, Hui Zhang, Dan Shu, Carlo Montemagno, Baoquan Ding, Jingyuan Li, and Peixuan Guo*

The significance of bionanomotors in nanotechnology is analogous to mechanical motors in daily life. Here the principle and approach for designing and constructing biomimetic nanomotors with continuous single-directional motion are reported. This bionanomotor is composed of a dodecameric protein channel, a six-pRNA ring, and an ATPase hexamer. Based on recent elucidations of the one-way revolving mechanisms of the phi29 double-stranded DNA (dsDNA) motor, various RNA and protein elements are designed and tested by single-molecule imaging and biochemical assays, with which the motor with active components has been constructed. The motor motion direction is controlled by three operation elements: (1) Asymmetrical ATPase with ATP-interacting domains for alternative DNA binding/pushing regulated by an arginine finger in a sequential action manner. The arginine finger bridges two adjacent ATPase subunits into a non-covalent dimer, resulting in an asymmetrical hexameric complex containing one dimer and four monomers. (2) The dsDNA translocation channel as a one-way valve. (3) The hexameric pRNA ring geared with left-/right-handed loops. Assessments of these constructs reveal that one inactive subunit of pRNA/ATPase is sufficient to completely block motor function (defined as $K = 1$), implying that these components work sequentially based on the principle of binomial distribution and Yang Hui’s triangle.

1. Introduction

The ubiquitous, intriguing nanomachines in living organisms have inspired numerous biomimetic strategies and human achievements. Biological macromolecules of DNA, RNA, and proteins are essential and powerful chemical building blocks of all organisms based on their intrinsically defined features at the nanometer scale.\cite{1,2} Construction

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of self-propelling nanomotors has been a popular subject in the field of nanotechnology.\[^{[3–24]}\] The emerging field of nanotechnology has led to the advancement of biomaterials engineering and synthetic biology.\[^{[25–27]}\] Although the construction of biological nanomotors has been extensively studied,\[^{[28–31]}\] the key step to make these bionanomotors applicable in nanodevices and nanomachines is to functionalize these motors and control their continuous single-directional motion. Here we report one such principle and approach by designing and constructing an artificial biomimetic phi29 dsDNA packaging motor that can move continuously in one direction. This nanomachine is composed of a 3.6 nm dodecameric protein channel and six protein ATPases that are geared by six pRNA (packaging RNA) and driven by ATP (Figure 1).\[^{[28]}\]

The motion direction of the dsDNA was controlled by ATPase, connector, and pRNA systems. In the asce (additional strand catalytic E) ATPase superfamily, one well-conserved ATP domain contains one arginine finger motif along with the Walker A and Walker B motifs.\[^{[32–35]}\] Located in proximity to the γ-phosphate of the bound ATP in the adjacent ATPase subunit,\[^{[33,36–38]}\] arginine finger has been reported to be involved in the formation of the ATP activity pocket.\[^{[35,38–42]}\] Elucidation of the principle of how arginine finger mediates the sequential action of ATPase has shed light on the realization of controlling motor motion direction. Furthermore, the connector channel as a one-way valve for DNA advancement is another key factor for the control of the uni-directional movement of the biomotor. With the contribution of all three coaxial rings in the motor, the constructed motor, though tiny, is considered to be one of the most powerful biological motors ever constructed to date.

2. Results and Discussion

2.1. Screening of pRNA Sequences to Control Motor Motion

pRNA is an essential component of the motor.\[^{[43]}\] Six pRNA monomers form a hexameric ring through interactions between four bases in every two adjacent pRNA loops.\[^{[44]}\] A minimum length of 117 nucleotides in each pRNA is required. Each pRNA contains five single-base bulges, one three-base bulge, one bifurcation bulge, one bulge loop, and two stem loops (Figure 2). To select the RNA sequences with activity to gear the motor, 18 different hexamer pRNAs were constructed following the method reported previously.\[^{[44,45]}\] All these mutant pRNAs were competent in binding to the procapsid with an efficiency equal to that of the wild-type pRNA. However, incorporation of only one mutant, either pRNAdCCA (with CCA bulge deletion, Figure 2, red box) or pRNAtur (truncated at the 5′- and 3′-ends, Figure 2, blue box), into the hexameric loop completely obliterated the motor function in DNA packaging activity. The fact that one inactive pRNA is sufficient to block the function of the entire motor has been defined as $K = 1$.\[^{[46–48]}\] In binomial distribution, $K = 1$ for the hexametric RNA ring implies that six RNA molecules worked sequentially.\[^{[48–50]}\]

2.2. Screening of Connector Sequences to Control Motor Motion

The channel of the phi29 motor functions as a dodecameric ring and serves as a pathway for the dsDNA to enter the procapsid during the packaging process. The protein subunit of the connector was modified to screen for active mutants for motor construction. This was achieved by adjusting the inner loop of the connector protein, or by applying terminus extensions or truncations to the connector protein.

The inner loops of the phi29 connector are expected to be involved in the one-way traffic of DNA translocation during motor packaging (Figure 3A, left).\[^{[51,52]}\] A similar internal loop is also found in the portal protein of SPP1 (Figure 3A, right). Single-pore conductance assay has been utilized to test the performance of the phi29 connector channels. With the channel embedded and voltage applied across the membrane, the translocation of the negatively charged DNA through the channel can be detected. With the wild-type connector, DNA translocation events occurred only when the channel entrance faced the negative electrode.\[^{[52,53]}\] In contrast, DNA translocated through the channel from both directions when using the loop-deleted connector.\[^{[52,54]}\] The results strongly support that the internal loops of the connector are one of the key factors for the one-way traffic of dsDNA and
that the direction of DNA translocation through the motor can be controlled by tuning the internal loop of the channel protein.

Fusion of small peptides to the N-terminus or C-terminus of gp10 generated different gp10 extensions, namely gp10-Nhis, gp10-Nstrephis, and gp10-Cstrep, with the tobacco etch virus (TEV) cleavage site incorporated.[55] The mutant procapsid with N-terminal extensions (gp10-Nex) demonstrated a 100-fold reduction in virion assembly compared to the wild-type procapsid (Figure 3B,C). Removal of the extended sequence by TEV cleavage restored virion assembly activity of phi29. On the other hand, the procapsids with C-terminal extensions (gp10-Cex) were slightly impaired in assembly activity (tenfold drop) (Figure 3B,C). gp10-Ntrun with N-terminal residues 1–14 truncation and C-terminal strep tag extension was generated. To further define the sequence requirement for building the dodecameric architecture, residues 1–37 or 1–48 of gp10 were removed and tested. Residues 11–37 constitute one entire \( \alpha \)-helix running from the narrow end to the wide end of the connector, followed by residues 38–48 which form a \( \beta \)-sheet at the relaxed wider end of the connector. It was obvious that this \( \alpha \)-helix was intimately related to connector assembly. Its removal disrupted the balance and self-interaction of gp10 subunits in a connector, or worse, impaired gp10 protein folding which led to insoluble aggregates. In addition, truncation of the flexible region of C-terminal 25 residues generated procapsid-Ctrun with normal morphology but with its virion assembly completely blocked. We speculate that the C-terminal region plays an important role inside the procapsid to facilitate DNA packaging.

2.3. Screening of the Motor ATPase with Different Sequences of ATPase Pockets to Control Motor Motion

Previously, it has been reported that the phi29 DNA packaging motor adapts sequential action for its coordination among subunits.[49,56] More recently, this coordination has been shown to be mediated by the arginine finger motif in the gp16 ATPase of phi29.[42] Phi29 motor ATPase gp16 shares the common ATP activity domain typical of all members of
the ASCE superfamily,[57,58] including the very well-conserved motifs responsible for ATP binding and ATP hydrolysis,[59] which have been previously identified as Walker A[60] and Walker B motifs[61] respectively. Sequence alignment studies have further revealed a common arginine residue.[38,62–65] The alteration in arginine 146 severely impaired the ATP hydrolysis activity and motor DNA binding activity of gp16, as confirmed by EMSA (electrophoresis mobility shift assay) and capillary electrophoresis (CE) assay, which showed the ≈4-fold reduction in binding of gp16 arginine finger mutant with dsDNA compared to that of wild-type gp16 (Figure 4).

The presence of a non-covalent dimer of the ATPase gp16 has been shown by different approaches including EMSA, glycerol gradient ultracentrifugation, and native gel electrophoresis (Figure 4).[61] Dimer formation requires that at least one of the adjacent subunits can provide an intact arginine for inter-subunit interactions, since dimers were produced when the arginine-free ATPase was mixed with the wild type, which has an intact arginine, but not with the arginine-free ATPase (Figure 4). Furthermore, it was shown that one single inactive subunit of arginine 146-free ATPase was able to completely, other than partially, block the function of the entire motor[42] that is, \( K = 1 \) in binomial distribution as described above, which implies that six ATPase molecules worked sequentially.[46–48]

The hexameric structure of gp16 ATPase has been constructed with \( P. \) aeruginosa FisK (PDB ID: 2IUU) as a template[62] based on its predicted monomer structure using I-TASSER[67] software. The core structure of both the N-domain of gp16 and \( \beta \)-domain of FtsK with the length of 127 residues was aligned, and the RMSD of backbone carbon atoms in these core structures was calculated to be 3 Å. Considering the size of these two proteins and the protein structure comparison, an RMSD of 3 Å can effectively reflect that gp16’s N-domain adopts the same fold as FtsK’s \( \beta \)-domain.[68,69] The model showed that the position of arginine 146 in one ATPase subunit outstretches to the ATPase domain in a neighboring subunit (Figure 5), which agrees with our data showing the cooperative behavior in the hexameric ATPase ring. As demonstrated above, it is concluded that arginine finger serves as a bridge between single subunits to form non-covalent dimers.

### 2.4. ATP Binding and Hydrolysis Triggers Conformational Entropy Changes of ATPase with High or Low DNA Binding Affinity

ASCE proteins undergo a cycle of conformational changes upon ATP binding and hydrolysis with two major states: high or low affinity for the substrate.[70] In recent publications,[50,56,61,71] a similar model of sequential action for gp16 has been reported. Partial proteolysis treatment and tryptophan intrinsic fluorescence assays indicated a
conformational change in the gp16-ATP complex, specifically, a more constrained and less conformational entropy for gp16 before ATP binding. EMSA, capillary electrophoresis assays, and FRET assays showed stronger binding of gp16 to dsDNA in the presence of $\gamma$S-ATP (Figure 4), suggesting that the gp16/dsDNA complex is stabilized upon addition of the non-hydrolyzable ATP substrate.

As a dsDNA-dependent ATPase,[60,72,73] binding of the gp16/ATP complex to dsDNA resulted in ATP hydrolysis, leading to its second round of conformational entropy change to a low DNA-affinity configuration and subsequently to the release of dsDNA for its concomitant transfer to the adjacent downstream subunit. An increased ATP hydrolysis rate has been observed when the ATPase is bound to DNA, indicating a global structural alternation in the protein upon DNA binding/release.[50] Taken together, a mechanism of ATPase for dsDNA translocation is proposed (Figure 6) for constructions of active motors, showing that

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**Figure 4.** ATP binding and hydrolysis activity assay of gp16 arginine finger mutant and inter-subunit interactions of ATPase. A) Capillary electrophoresis assay for the binding affinity test of different gp16 with dsDNA. B) Interactions between gp16 arginine finger mutants with gp16 wild-type are shown by the band shift of both ATPase and DNA in the gel. (Green: mCherry channel; blue: eGFP channel; red: Cy5 channel.) C) Both dimers and monomers exist in gp16 ATPase rings. In 15%–35% glycerol gradient, one peak for eGFP-gp16 R146A (a) and two peaks for eGFP-gp16 wild-type (b) were observed after parallel ultracentrifugation, indicating that dimer formation is interrupted by the mutation of arginine finger. The fractions derived from the gradient have been applied to EMSA (c) and in vitro assembly activity assay (d), confirming the formation of dimers mediated by arginine finger. The isolated gp16 dimer fraction (Fr. 18) showed significantly reduced activity compared to the monomers (Fr. 22) (d), supporting the previous finding that the addition of fresh gp16 monomer is required for re-initiating DNA packaging intermediates.[66]
the energy consumption status corresponds to the inter-subunit communication primed by the arginine finger. The resulting asymmetrical hexameric intermediate was supported by many other hexameric ATPase systems based on structural computation, X-ray diffraction, and Cryo-EM imaging (Figure 6).[74–78] The results provide clues as to why the hexameric ATPase gp16 of phi29 and gp17 of T4 was previously interpreted as having a pentameric configuration by cryo-EM.[79,80] With two adjacent ATPase subunits interacting with each other and forming a closer dimer configuration, the asymmetrical hexamer could be mis-analyzed as a pentamer after reconstruction by averaging numerous images as in Cryo-EM.

2.5. Single-Molecule Real-Time Imaging of the Biological Active Motor with Continuous Motion

The motion of the constructed artificial ATPase motor without the dodecameric channel and RNA ring was confirmed by single-molecule fluorescence imaging (Figure 7, Movie 1). Purified ATPase was labeled with Cy3 fluorescent dye and incubated with DNA, which was tethered between two polylysine beads. The motion of fluorescent spots representing the ATPase complexes was observed along the DNA chains through real-time recording, which confirmed the translocating activity of ATPase along dsDNA. Next, the successful construction of the artificial motor containing

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**Figure 5.** Illustration of inter-subunit interaction inside gp16 ATPase. Gp16 ATPase hexameric ring was constructed (left panel), and the interaction between two adjacent subunits (right panel) has been shown with the arginine finger highlighted in the red sphere and Walker domain (represented by E119 residue in Walker B domain) highlighted in the blue sphere. The interaction of arginine finger with the upstream adjacent subunit is supported by the relative location of the related domains.

**Figure 6.** A) ATPase sequential action mediated by arginine finger. ATPase coordination with a series of conformational changes during DNA binding and ATP hydrolysis as regulated by arginine finger, resulting in the asymmetrical configuration of ATPase. The asymmetrical structures have also been found in many other biomotors, including B) V1-ATPase[74] and C) MCM2-7 protein (EM accession: EMD-5429).[79]
both dodecameric ring and RNA hexamer to drive dsDNA was tested by fluorescence imaging in real time. DNA translocation was recorded through the biomotor constructed with all active motor components. The functional motor was stalled using non-hydrolyzable γS-ATP, isolated and then attached to a slide. The distal end of the dsDNA was labeled with a biotin moiety and attached to a fluorescent streptavidin-coated microsphere. After addition of ATP, the continuous motion of the motor was resumed and observed by real-time fluorescence microscopy through epi-illumination (Figure 8). Analysis of the motion in x, y, and z dimensions showed that the swing distance of the microsphere was reduced as the motion process was close to the end of packaging. As a control, the microsphere exhibited a mainly linear and static Brownian motion when the stalled motor was not restarted, indicating that the constructed motor was functional in continuous DNA translocation with a one-way direction (Figure 8, Movie 2). Single-directional motion of the motor was also detected in real time using a horizontal setup with biomotors assembled with the procapsids which confirmed the successful construction of the artificial motor with the control of motion direction.

3. Conclusion

Based on the one-way revolving mechanism of the phi29 dsDNA packaging motor, we have successfully constructed phi29 biomimetic nanomotors with altered and modified pRNA, ATPase, and a protein channel with single-directional motion. Arginine finger bridges two adjacent ATPase subunits to form a non-covalent dimer, resulting in an asymmetrical hexameric complex. Single-directional motion of the dsDNA was controlled by three sets of operation elements: (1) the asymmetrical hexameric ATPase containing one dimeric and four monomeric subunits that hold an ATP-interacting domain to regulate alternative DNA binding and also push an arginine finger to control the sequential action; (2) the dsDNA translocation channel that serves as a one-way valve to ensure single-directional advancement and to prevent the reversal of the dsDNA; (3) the hexameric pRNA ring geared with their left and right-hand loops. Assessment of the resulting constructs revealed that one inactive subunit of pRNA or ATPase is sufficient to completely, other than partially, block the function of the entire motor. The finding of $K = 1$ implies sequential action with single-directional motion, based on the criterion of binomial distribution and Yang Hui’s triangle.

4. Experimental Section

Cloning, Mutagenesis, and Protein Purification: The engineering of eGFP-gp16 and the purification of the gp16 fusion protein have been reported previously. The eGFP-gp16 mutant R146A and mCherry-gp16 mutant R146A were constructed by introducing mutations in the gp16 gene (Keyclone Technologies).

Glycerol Gradient Ultracentrifugation: 50 μL of eGFP-gp16 (500 μg mL⁻¹) were dropped onto 5 mL linear 15%–35% glycerol gradients in TMS buffer. After centrifuging at 35 000 rpm in a SW55 rotor at 4 °C for 22 h, the gradients were collected into 31 fractions from bottom to top and measured using a plate reader under 488 nm excitation before being applied to in vitro assembly assay. EMSA: Fluorescently tagged protein that facilitates detection and purification was shown to possess similar assembly and packaging activity as compared to wild-type. The EMSA method has been described previously. The gp16 mutants or wild-type were mixed with 33bp Cy5-dsDNA in the presence or absence of ATP and γS-ATP. Samples were incubated at ambient temperature for 20 min and then loaded onto a 1% agarose gel (44.5 × 10⁻³ M Tris, 44.5 × 10⁻³ M boric acid) and electrophoresed at 4 °C for around 1 h at 8 V cm⁻¹. The eGFP-gp16, mCherry-gp16, and Cy5-DNA samples were analyzed by a Typhoon FLA 7000 (GE Healthcare) using 473, 532, and 635 nm excitation wavelengths for GFP, mCherry, and Cy5, respectively.

CE Experiments to Determine Ratio of gp16 to Bound dsDNA: CE experiments were performed on a Beckman MDQ system equipped with double fluorescence detectors (at 488 and 635 nm excitation wavelengths). A bare borosilicate capillary with a total length of 60 cm and a 50 μm inner diameter was used. Assay conditions contained separation buffer of 50 × 10⁻³ M Tris–HCl, 100 × 10⁻³ M
sodium borate at pH 8.00, $5 \times 10^{-3}$ M MgCl$_2$, 10% PEG 8000 (w/v), 0.5% acetone (v/v), $3 \times 10^{-6}$ M eGFP-gp16 monomer, and variable amounts of ATP and DNA.

In Vitro Assembly Assay: Purified in vitro components, including prohead-connector, pRNA, genome DNA, ATPase, and tail proteins were mixed and subjected to virion assembly assay as previously described.[29] Briefly, newly assembled infectious virions were inoculated to Bacillus bacteria and plated. Activity was expressed as the number of plaques formed per volume of sample (pfu mL$^{-1}$).

Different fractions of samples were isolated from the glycerol gradient and added into the system for their activity test.

Observation of gp16 Motion: Double-stranded lambda DNA (48 kbp) was tethered between two polylysine coated 4 μm silica beads.[83] The dsDNA was bound between beads by back-and-forth infusion of DNA over the beads for 10 min; binding occurred as a result of charge–charge interactions. The back and forth motion of DNA over the polylysine beads stretched the DNA taut and lifted the chain above the surface by the 4 μm silica beads as visualized under the microscope. The incident angle of the excitation beam in objective-type TIRF (total internal reflection fluorescence) was adjusted to a sub-critical angle in order to image the samples a few micrometers above the surface, yielding a low fluorescence background.[83] To-Pro-3 was used to confirm the formation of the DNA tethers. After the DNA tether was formed, a 30 μL mixture with a final concentration of $1 \times 10^{-9}$ M Cy3-gp16 with $100 \times 10^{-9}$ M unlabeled gp16 in buffer B ($25 \times 10^{-3}$ M Tris, pH 6.1, $25 \times 10^{-3}$ M NaCl, $0.25 \times 10^{-3}$ M MgCl$_2$) was infused into the sample chamber for binding to the stretched DNA. After 30 min incubation, 30 μL of a solution containing anti-photobleaching reagents[81] was infused into the chamber in order to prevent photobleaching of less photostable fluorophores and to detect binding. Movies were taken after the chamber was washed with buffer C ($25 \times 10^{-3}$ M Tris, pH 8, $25 \times 10^{-3}$ M NaCl, $0.25 \times 10^{-3}$ M MgCl$_2$). A comparison was made of washing with buffer C, with and without $20 \times 10^{-3}$ M ATP. Since the DNA has been fixed by charge interactions and the protein fixed by binding affinity to the tethered DNA, washing does not remove pertinent material. Sequential images were acquired with a 0.2 s exposure time at an interval of 0.22 s, with a laser of 532 nm for excitation. The movies were taken for about 8 min, or until the Cy3 fluorophores lost their fluorescence due to photobleaching. Image J software was utilized to generate kymographs to show the displacement of the Cy3-gp16 spots along the DNA chains.
Direct Observation of DNA Translocation: The stalled packaging intermediates containing biotinylated DNA were prepared by using non-hydrolyzable γ3-ATP.[66] The intermediates were then immobilized through IgG probe head antibody to perfusion chambers built from glass slides and coverslips. The fluorescent streptavidin microspheres (0.56 μm) were bound to the protruding, biotinylated DNA end of the intermediates. After restarting the packaging reaction by adding gp16 and ATP, an individual DNA-packaging event was observed. Epi-illumination was used, and sequential images were recorded.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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