Strand and nucleotide-dependent ATPase activity of gp16 of bacterial virus phi29 DNA packaging motor

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A B S T R A C T
Similar to the assembly of other dsDNA viruses, bacterial virus phi29 uses a motor to translocate its DNA into a procapsid, with the aid of protein gp16 that binds to pRNA 5′/3′ helical region. To investigate the mechanism of the motor action, the kinetics of the ATPase activity of gp16 was evaluated as a function of DNA structure (ss- or ds-stranded) or chemistry (purine or pyrimidine). The $k_{cat}$ and $K_m$ in the absence of DNA was 0.016 s$^{-1}$ and 351.0 μM, respectively, suggesting that gp16 itself is a slow-ATPase with a low affinity for substrate. The affinity of gp16 for ATP was greatly boosted by the presence of DNA or pRNA, but the ATPase rate was strongly affected by DNA structure and chemistry. The order of ATPase stimulation is poly d(pyrimidine) > DNA > poly d(purine), which agreed with the order of the DNA binding to gp16, as revealed by single molecule fluorescence microscopy. Interestingly, the stimulation degree by phi29 pRNA was similar to that of poly d(pyrimidine). The results suggest that pRNA accelerates gp16 ATPase activity more significantly than genomic dsDNA, albeit both pRNA and genomic DNA are involved in the contact with gp16 during DNA packaging.

Introduction

Most linear double-stranded (ds) DNA bacteriophages translocate their genomic DNA into a pre-assembled protein shell, known as a procapsid, to near-crystalline density. Due to the limitation of the spatial capacity of procapsids, these viruses have developed unique ATP-driven viral DNA packaging motors to complete such an energetically unfavorable DNA packaging process (Black, 1989).

The DNA packaging motor of many dsDNA bacteriophages consists of a dodecameric protein portal, also called the connector (Simpson et al., 2000; Ibarra et al., 2000), and two nonstructural components that comprise the packaging enzyme complex with certain characteristics typical of an ATPase (Guo et al., 1987). Mostly, the large subunit is responsible for procapsid binding, genome maturation (cleaving concatemeric DNA) and DNA translocation, while the small subunit recognizes the unique sequences on its own genomic DNA to employ the large subunit to assemble into an active terminase complex. The packaging motor of phi29 involves four essential components: terminal protein gp3, the 12-subunit connector (Gausch et al., 2002; Simpson et al., 2001; Simpson et al., 2000; Ibarra et al., 2000), the DNA packaging protein gp16 (Guo et al., 1987), and the packaging RNA (pRNA) hexamer (Guo et al., 1998; Zhang et al., 1998).

Previously, it was found that gp16 binds dsDNA without discrimination of phi29 genomic DNA versus non-phi29 DNA (Lee and Guo, 2006). It was also revealed that gp16 specifically docks 5′/3′ paired ends of pRNA on the packaging motor. This procapsid/pRNA complex then recruits the gp16 to assemble into an active packaging motor for the further interaction with genomic DNA or for DNA translocation. Thus, gp16 serves as both a linkage between pRNA and DNA and as an essential DNA-contacting component during DNA translocation (Lee and Guo, 2006).

The ATPase activity of gp16 can be stimulated either by pRNA or by DNA. Similarly, procapsids with pRNA stimulate the ATPase activity of gp16 ten-fold, as compared with the lack of stimulation by procapsids alone. The maximum stimulation has been observed only when all packaging motor components, including pRNA, procapsid, gp16, and DNA-gp3, were present (Guo et al., 1987; Shu and Guo, 2003a; Grimes and Anderson, 1990, 1997; Ibarra et al., 2001). These stimulation effects were also found in other viral packaging enzymes. For instance, in phage lambda, each subunit of terminase possesses distinct ATPase activities (Hwang et al., 1996). The small subunit, gpNu1, is responsible for DNA recognition and DNA cutting, and shows DNA-dependent low affinity ATPase activity ($K_m = 469$ μM with DNA). Pac-ATPase subunit of T3 terminases also shows viral DNA-dependent ATPase activity, while the other subunit, termed Non-pac ATPase, was stimulated by non-packagable DNA (i.e., single-stranded or circular) or RNA (non-specific) (Morita et al., 1993). These individual ATPase activities also can be stimulated by the interaction with other components. Formation of holoenzyme increases their ATPase activity. In SPP1, interactions between two or three G2P and one G1P, assembled into...
hologenezymes, showed the enhanced ATPase activity (Camacho et al., 2003). The ATP consumption in DNA packaging, in the defined in vitro DNA packaging system of phi29, was estimated to be that every ATP hydrolysis corresponds to 2 base pairs movement of DNA (Guo et al., 1987). In addition, the recent single molecule studies revealed that the phi29 packaging motor has shown an initial rate of 100 base pairs per second with an extra load of 2.2 \mu m polystyrene beads on the DNA. It also showed that the force corresponds to 57 pN (Smith et al., 2001; Fuller et al., 2007), which makes it one of the most powerful biomonomotors constructed to date. Although it seems obvious that gp16 possesses a consensus ATP-binding sequence (Guo et al., 1987) and contributes as part of the ATPase complex to provide the packaging energy, no biochemical kinetic study has been reported yet on the ATPase activity of gp16 in phi29 and on the correlation to the stimulation effect of DNA binding by gp16. It is critical to understand how phi29 packaging motor gains the driving force from ATP hydrolysis by investigating ATPase activity of gp16 and its stimulation through the interaction with DNA substrate.

Conventional thin layer chromatography (TLC) has been used to separate the inorganic phosphate (P_i) released from the hydrolysis of \( \gamma^{32P} \) ATP by gp16 (Huang and Guo, 2003a; Shu and Guo, 2003b). However, due to the low reproducibility in autoradiography analysis and the poor resolution in early stage events of ATP hydrolysis, it is not an ideal method for kinetic studies. It is essential to observe the hydrolysis events during the early stages of reaction to accurately calculate the initial velocity. For those reasons, an MDCC-PBP (N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide-phosphate binding protein system has been employed. The phosphate binding protein (PBP) of Enterobacteriaceae is a periplasmic protein, which is produced under low phosphate conditions and transports P_i into the cytoplasm (Brune et al., 1998). To be used as a P_i probe, a cysteine was introduced at position 197 on the PBP, and was selectively labeled with a fluorophore (MDCC). The resultant labeled protein (MDCC-PBP) binds P_i tightly (K_i \sim 0.1 \mu M) and gives an increase in fluorescence at 460 nm. MDCC-PBP has been used to investigate the kinetics and role of P_i release in a number of systems that involve ATP hydrolysis (He et al., 1997; Brune et al., 1994). In this report, we determined the kinetic parameters for the ATPase activity of gp16 by using the fluorescent phosphate sensor system, MDCC-PBP. We also investigated the effect of random sequence dsDNA of 54 bp was very similar to that by a homopolymeric dsDNA (poly dA-poly dT) with a similar size K_m of 351.0 \pm 34.0 \mu M and k_cat/K_m of 45.0 M^{-1} s^{-1}, suggesting that gp16 itself is a slow-ATPase with low affinity for substrate (Table 1). This is the first kinetic measurement of the ATPase activity of gp16.

Comparison of the stimulation effect between double-stranded and single-stranded DNA on ATPase activity of gp16

DNA is indispensable for the force-generating ATP hydrolysis process of the viral DNA packaging enzymes as investigated in phi29 (Guo et al., 1987), lambda (Wei et al., 1992; Hwang et al., 1996), T4 (Baumann and Black, 2003; Alam and Rao, 2008) and T3 (Morita et al., 1993). It has been reported that gp16 interacts with DNA in a sequence independent manner (Lee and Guo, 2006; Guo and Lee, 2007). To investigate the effect of dsDNA chemistry and structure on the ATPase activity of gp16, dsDNA fragments with a size of 25, 54, and 160 bp were tested in the defined ATPase assay system (Table 1). The presence of dsDNA affected both K_m and k_cat of the ATPase activity of gp16 so that K_m/K_i was increased at least 10 times greater than in the absence condition (Table 1). The rates of ATP hydrolysis activity (k_cat) of gp16 were similar for these differently sized dsDNA fragments. As well, the K_m for the differently sized DNAs were similar. Furthermore, stimulation by random sequence dsDNA of 54 bp was very similar to that by a homopolymeric dsDNA (poly dA-poly dT) with a similar size.

**Results and discussion**

**Determination of the K_m and k_cat for gp16**

To determine the kinetic parameters for ATP hydrolysis of gp16, the reaction was initiated by adding ATP and gp16 into a buffer containing MDCC-PBP as an indicator for the release of P_i (Fig. 1A). The results revealed a relationship of initial velocity V_{init} (\mu M s^{-1}) to concentration of gp16 (\mu M) (Fig. 1B). ATP hydrolysis was not detectable when the concentration of gp16 in the reaction mixture was under 0.6 \mu M, while a linear relationship existed within a range of 0.5 and 2 \mu M of gp16. From this observation, 1 \mu M of gp16 was used in the remainder of the studies and the concentration of MDCC-PBP was optimized at 2 \mu M, enough to cover the released P_i from ATPase hydrolysis. To examine the steady-state parameters of ATP hydrolysis by gp16, the assay was performed over a range of ATP concentrations. The ATP hydrolysis assay showed a linear relationship of increasing V_{init} to increased concentration of ATP (Fig. 2), which implies that gp16 is a Michaelis–Menten-type enzyme. The catalytic rate of ATP hydrolyzing reaction by gp16 (k_cat) was determined by linear regression plotting based on the Michaelis–Menten equation. The k_cat of gp16 was 0.016 \pm 0.002 s^{-1} with a

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**Fig. 1.** Determination of the ATPase activity of gp16. (A) Example data showing how to determine an initial velocity from the burst of P_i as released from the ATPase reaction by gp16. The released P_i is scavenged by MDCC-PBP, which are excited at 425 nm and the emission was detected at 464 nm by fluorometer. The initial velocity V_{init} (dashed line) was described as \mu M s^{-1}. (B) Optimization of the concentration of gp16 under 1 \mu M of ATP and 2 \mu M of MDCC-PBP. Each data points represent the initial velocity of the ATPase activity in the given concentration of gp16 that are expressed as linear scale.
of 50 bp (Table 1). These results confirm our previous findings (Lee and Guo, 2006) that gp16 randomly binds dsDNA in a non-sequence specific manner and indicate that no specific sequence is required for the stimulation of ATPase activity of gp16.

The stimulation effect of 54 bp dsDNA on the ATPase activity of gp16 was further compared to that of 50 bases homopolymeric ssDNA, such as poly dT and poly dA. The use of these homopolymers avoids possible interference from partially double-stranded regions or other secondary or tertiary structures. The results showed that the slope for dsDNA lies near the slope for poly dA, with the interesting finding that poly dT stimulates the ATPase activity of gp16 most efficiently (Fig. 2). The $k_{cat}$ for poly dT was higher than that for dsDNA. However their $k_{cat}/K_m$ were close, since in its interaction with gp16, dsDNA had a lower $K_m$ and $k_{cat}$ than poly dT (Table 1). To the contrary, $k_{cat}/K_m$ for poly dA was lower than that of dsDNA or poly dT (Table 1), which means that the dsDNA or poly dT binding to gp16 increased their specificities to ATP. In addition, it suggests that the stimulation of the ATPase activity of gp16 caused by dsDNA or poly dT binding was due to an increase in the catalytic rate of the ATPase activity of gp16 together with an increase of the specificity for ATP.

**Comparison of the stimulation effect of pRNA and genomic DNA on ATPase activity of gp16**

It has been reported that the ATPase activity of gp16 was stimulated by pRNA (Grimes and Anderson, 1990; Ibarra et al., 2001; Shu and Guo, 2003a). It has also been reported that gp16 serves as a bridge between pRNA and genomic DNA by binding to both pRNA and dsDNA (Lee and Guo, 2006). However, the strength of the stimulation effect has not been quantified. Kinetic analysis revealed that the slope for pRNA-dependent ATPase activity was close to that of poly dT (Fig. 2). $k_{cat}$ and $K_m$ in the presence of the pRNA were similar to that of poly dT, and far apart from that of dsDNA (Table 1). This indicates that pRNA is the preferred structure over dsDNA in stimulating the ATPase activity of gp16.

**Comparison of the stimulation effect between poly dT and other poly-nucleotides on ATPase activity of gp16**

To further verify the effect of ssDNA on the ATPase activity of gp16, poly dG and poly dC were compared with poly dA and poly dT. The poly d(pyrimidine), poly dT and poly dC, was a more effective stimulator than poly d(purine), poly dA and poly dG (Fig. 2), $k_{cat}$ for poly dT and poly dA were 0.141 ± 0.017 and 0.044 ± 0.01 s$^{-1}$, respectively (Table 1), indicating that poly dT stimulated the ATPase rate of gp16 at least 3 times higher than poly dA and almost 9 times higher than gp16 itself. In addition, $k_{cat}/K_m$ for poly d(purine) was at least 2 times lower when it was compared with poly d(pyrimidine). However, $K_m$ was nearly unaffected by the presence of ssDNA, while ssDNA mostly changed $k_{cat}$. This data indicates that the stimulating effect of ssDNA on the ATPase activity of gp16 depended on its chemistry (type of nucleotide) (Table 1).

The sensitivity of gp16 to the type of DNA leads to a more important question of whether the structure of DNA plays a significant role in interacting with other motor components during translocation. If this is the case, how the helical nature of dsDNA is involved in the mechanism of motor motion would be of interest. Recently, it was proposed that one of the motor components, the connector, does not rotate during DNA translocation (Baumann et al., 2006; Hugel et al., 2007). Nevertheless, most DNA packaging models of phi29 favor a rotary motor mechanism. According to this model, the helical nature of dsDNA plays an essential role so that the different pitch and depth in different DNA types might affect the interaction with gp16. In alternative packaging models, which are predominantly based on the alignment of the packaging enzymes with recognition sites on the DNA, higher tolerance toward structural alterations would be expected (Simpson et al., 2000; Guasch et al., 2002). In these models, the binding sites on the DNA need to be sufficiently intact for the interaction with the packaging enzyme. Studies on the packaging activity for structurally modified DNA substrates might help to understand specific interaction between gp16 and genomic DNA during packaging, which can be useful to turn the motor off (and potentially back on) at specific sites on the DNA. Indeed, changing the DNA from helix to single strand may serve as a mechanism to completely stop the translocation of phi29 DNA (Moll and Guo, 2005). This speculation was supported by the finding that the stimulating effect of homopolymeric dsDNA (poly dA:poly dT) containing 50% purine and 50% pyrimidine lies between those of poly d(purine) and poly d(pyrimidine), and that the poly dA:poly dT made no difference in the stimulating effect from random dsDNA also containing 50% purine and 50% pyrimidine (Fig. 2). Whether the observation that only 50% (pyrimidine, but not purine) of the nucleotides had a strong effect on stimulating the ATPase activity of gp16 can be correlated with the previous findings that, on average, one ATP consumption packages two base pairs of substrate DNA (Guo et al., 1987; Morita et al., 1993; Chemla et al., 2005) remains to be investigated. In addition, the finding that the DNA frequently slipped out of the procapsids, as the DNA was packaged during the packaging process (Smith et al., 2001), might have a certain relationship with the sequence on the DNA-gp3. Since our single molecule photoimaging data shows that poly dT binding to gp16 was stronger than poly dA, it can be inferred that a purine-rich region on the DNA might decrease the binding affinity of

![Fig. 2. Determination of kinetic parameters for an intrinsic ATPase activity of gp16. The rate of ATPase activity of gp16 ($k_{cat}$) was determined by linear regression plotting (Hanes–Woolf plot) based on the Michaelis–Menten equation. $k_{cat}$=0.016±0.002 s$^{-1}$, $K_m$=351.0±34.0 μM (n=7). Inset box shows the stimulation effect of DNA on the ATPase activity of gp16 depending on the chemistry and structure. Stimulated $V_{max}$ of gp16 ATPase activity by single-stranded poly dT (closed circles) and poly dA (closed triangles) were compared to that by double-stranded 54 bp DNA (open squares) and pRNA (open diamonds). Open circles represent the ATPase activity of gp16 in the absence of DNA.](image-url)

**Table 1** Kinetic data for ATPase activity of gp16

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Sequence</th>
<th>Size (bp)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (ATP, μM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>No nucleic acid</td>
<td>–</td>
<td>–</td>
<td>0.016±0.002</td>
<td>351.0±34.0</td>
<td>45.0</td>
<td>7</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Random</td>
<td>25</td>
<td>0.041±0.001</td>
<td>90.1±11.0</td>
<td>455.0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Random</td>
<td>54</td>
<td>0.059±0.003</td>
<td>90.0±33.4</td>
<td>654.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Random</td>
<td>160</td>
<td>0.080±0.003</td>
<td>66.9±7.8</td>
<td>896.9</td>
<td>3</td>
</tr>
<tr>
<td>ssDNA</td>
<td>poly dA:poly dT</td>
<td>50</td>
<td>0.055±0.003</td>
<td>109.2±11.9</td>
<td>501.7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>poly dA</td>
<td>50</td>
<td>0.044±0.001</td>
<td>128.4±28.0</td>
<td>339.1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>poly dC</td>
<td>25</td>
<td>0.061±0.007</td>
<td>183.1±5.3</td>
<td>332.2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>poly dG</td>
<td>50</td>
<td>0.139±0.007</td>
<td>197.8±17.7</td>
<td>704.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>poly dT</td>
<td>50</td>
<td>0.141±0.007</td>
<td>177.7±31.8</td>
<td>818.1</td>
<td>6</td>
</tr>
<tr>
<td>RNA</td>
<td>Wild type pRNA</td>
<td>117</td>
<td>0.122±0.004</td>
<td>165.5±24.2</td>
<td>735.2</td>
<td>7</td>
</tr>
</tbody>
</table>

*K_m refers to the ATP concentration at 1/2 $V_{max}$ in Michaelis–Menten analysis.*
the packaging motor against DNA. This loose interaction between the DNA and gp16 in the packaging motor gives rise to a slipping phenomenon due to the highly built up pressure inside the capsid during packaging. This theory is based on the assumption that gp16 interacts with one strand out of the duplex DNA during DNA translocation.

Comparison of gp16 binding affinity among double-stranded DNA, single-stranded DNA, and pRNA using single molecule fluorescence microscopy

Based on the result that the poly d (pyrimidine) stimulates the ATPase activity of gp16 stronger than the poly d(purine), it is central to investigate the binding ability of gp16 to explain the cause for such a distinctive effect. Single molecule fluorescence microscopy was employed to compare their binding affinity for gp16. Gp16 was first immobilized on the quartz surface pre-coated with anti-gp16 IgG. Binding of the fluorescent-ssDNA or pRNA was directly imaged using SMDV-TIRF single molecule dual viewing system (Shu et al., 2007; Zhang et al., 2007). Each visualized spot represents one single DNA molecule that binds to gp16, as confirmed by single molecule photobleaching technique (Shu et al., 2007; Zhang et al., 2007). It was found that Cy5-poly dT was bound to gp16 even at 10 pM, while Cy5-poly dA bound to gp16 was not observed at the lower concentration of 10 nM. However, dsDNA (Cy5-poly dT:Cy3-poly dA) showed a stronger binding affinity than poly dA but a weaker binding affinity than poly dT (Fig. 3A). In addition, Cy3-pRNA binding to gp16 was greater than both Cy3-poly dA and dsDNA (Cy5-poly dT:Cy3-poly dA). The order of the binding strength was in agreement with the order of the ATPase activity stimulated by the respective DNA or pRNA. Mean fluorescence intensity analysis showed that the difference of gp16 binding intensity between poly dT and poly dA is far greater than their difference in stimulating the ATPase of gp16 (Fig. 3B). Nevertheless, these results suggest that gp16 binds poly dT, representing poly d(pyrimidine), or pRNA with higher affinity compared with poly dA, representing poly d(purine), or dsDNA. The agreement between the order of the binding to gp16 and the stimulation effect on the ATPase activity of gp16 implies that the enhancement of ATPase stimulating activity was a result of their higher binding affinity for gp16.

Our data reveals that DNA and pRNA enhanced both the binding affinity and the catalytic activity of gp16 on ATP. It also reveals that this enhancement was correlated to DNA or pRNA binding to gp16 (Fig. 3A and Table 1). The following model might explain why DNA or pRNA binding to gp16 affects the ATPase function of gp16. DNA or pRNA binding to gp16 could trigger a conformational change of gp16. Such structural alteration will activate a catalytic site on gp16 to enhance its affinity to ATP, thus elevating ATPase activity. Our data also shows that

Fig. 3. Binding preference of gp16 to DNA observed by fluorescence single molecule imaging microscopy. (A) Fluorescence images of fluorescent DNA bound to gp16-immobilized surface. The range of either Cy5-poly dT or Cy3-poly dA was incubated inside gp16-immobilized microchamber. For the doubles-stranded poly dA-poly dT, Cy3-poly dA and Cy5-poly dT were hybridized to anneal each other and purified from 12% native PAGE. Phi29 Cy3-pRNA binding to gp16 was compared to the DNA bindings as the same manner. (B) Binding affinity comparison of gp16 between Cy5-poly dT (closed circles) and Cy3-poly dA (open circles) by plotting their mean fluorescence intensities over the concentrations of the fluorescent DNAs. The selected area is 37 μm × 77 μm.
both the ATP binding and hydrolysis by gp16 was correlated with DNA structure and chemistry, which however did not affect the $K_m$ in gp16 ATPase kinetic analysis. These observations raise some challenging questions. Does gp16 or the phi29 packaging motor involve single-stranded DNA during the motor assembly or packaging process? Does the phi29 double-stranded genome partially separate into a single-stranded form in some contact region of the packaging motor due to the involvement of any unknown functions such as gyrase, helicase, or topoisomerase during DNA translocation? The previous findings that nicked phi29 DNA can still be packaged (Moll and Guo, 2005) as well as similar phenomena in other phages including T3 (Fujisawa et al., 1987), T5 (Hayward and Smith, 1972), T7 (Khan et al., 1995) and Rhodopsseudomonas sphaeroides bacteriophage RSI (Donohue et al., 1985) do not support this argument. Any relationship between ATPase activity of gp16 and the DNA packaging process depending upon the structure and chemistry of DNA still needs to be answered.

Experimental procedures

Materials

Gp16 was prepared as previously described (Huang and Guo, 2003a; Lee and Guo, 2006). Gp16 was purified to homogeneity and tested for its full DNA packaging activity in the in vitro phi29 assembly system (Lee and Guo, 1995) before the ATPase assay. Poly d(N) was synthesized to be 50 bases (except 25 bases of poly dC) (IDT Inc., USA). For fluorescence single molecule microscopy, Cy5 or Cy3 was conjugated on the 5′-end of poly d(A or T) (IDT Inc., USA). MDCC-PBP was purchased from Invitrogen, USA. All dsDNAs used in our experiments were expressed with the prefix “ds-”, while the prefix “ss-” was used to refer to the single-stranded DNA. Poly d(N) refers to homopolymeric single-stranded DNA and poly dA:poly dT refers to the hybridized double-stranded heteroduplex DNA.

Measurement of ATPase activity by fluorometry

Purified gp16 was mixed with MDCC-PBP in H2O (20 mM HEPES, pH 8.0, 100 mM NaCl and 10 mM MgCl2) containing 0.2 μM of purine nucleoside phosphorylase (PNPase) (Sigma), 0.04 mM 7-methyl guanosine (Sigma) and 6% glycerol and then placed in a quartz microcuvette positioned in a Cary Eclipse spectrophotometer (Varian, Inc.). After the background fluorescence was checked for 10 seconds, ATP solution was added to initiate the ATP hydrolysis reaction. P1 was scavenged by MDCC-PBP when released from the ATPase reaction. The P1-bound MDCC-PBP was excited at 425 nm wavelength and the emitted fluorescence was monitored at 464 nm wavelength with 0.25 second time intervals under the control of the Cary Eclipse analysis software (Varian, Inc.). The reaction was terminated by the addition of 250 μM of KH2PO4 (pH 6.5).

Kinetic study of ATPase activity of gp16

Initial fluorescence represents the intensity of free MDCC-PBP ($I_h$). The ATP hydrolysis reaction was initiated by the addition of ATP solution (pH 7.0). The released $P_1$ was proportional to the burst of fluorescence as an observed intensity at time $t$ ($I_{obs}(t)$). The amount of MDCC-PBP bound to $P_1$ ($X_{b0}(t)$) was calculated by subtracting the intensity of free MDCC-PBP ($I_h$) from the observed intensities at time $t$ ($I_{obs}(t)$), and the difference was divided by the difference between the saturated $P_1$-bound MDCC-PBP ($I_{satura}$) in the presence of excess $P_1$ and free MDCC-PBP ($I_h$). The equation can be expressed as $X_{b0}(t) = (I_{obs}(t) - I_h)/[I_{satura} - I_h]$. The initial velocities ($V_{init}$) were calculated as the linear slope of released $P_1$ (μM) from ATPase activity of gp16 over time (s). Hanes–Woolf plot was used to determine kinetic parameters during steady-state ATP hydrolysis of gp16 by rearranging Michaelis–Menten equation ($V_{init} = V_{max}[ATP]/(K_m + [ATP])$ to be $[ATP]/V_{init} = [(ATP) + K_m]/V_{max}$. In the equation, the ratio of ATP concentration ([ATP], μM) to $V_{max}$ (μM s−1) is plotted over [ATP], in which data is expressed as a straight line of slope $1/V_{max}$ and a y-intercept of $K_m/V_{max}$. The linear regression analysis of data was performed using the OriginPro version 7.5 software (Origin Lab Corp.).

Fluorescence single molecule microscopy for DNA binding to gp16

The quartz surface of the perfusion chamber (15 μl internal volume) was coated with 100 μg/mL of anti-gp16 rabbit IgG (Proteintech Group, Inc) in HMS incubated overnight at 4°C. The gp16 (100 nM) diluted in HMS was infused into the microchamber and incubated for 30 min at RT for Ab-Ag binding. The unbound gp16 was washed using HMS. Each Cy5- or Cy3-labeled DNA in HMS was then incubated in the gp16-immobilized microchamber for 20 min at RT. After washing with HMS, an oxygen depletion solution containing glucose oxidase, catalase, β-d-glucose, and 2-mercaptoethanol (Ha et al., 2002) was infused inside the chamber before observation to reduce the effect of photobleaching of fluorescent materials. Observation of bound fluorescent DNA to gp16 was conducted by the total internal reflection fluorescence (TIRF) imaging technique as previously described (Shu et al., 2007; Zhang et al., 2007). An Olympus IX71 inverted microscope with 60× oil immersion objective (Planoapo, 60 ×, NA = 1.4) was used, and an extra 1.6× magnification lens was inserted for the further magnification of the obtained image. A laser beam with a 638 nm wavelength was used to excite Cy5-DNA, and a 532 nm wavelength was used to excite Cy3-DNA or Cy3-pDNA. The beam size is about 80 μm × 180 μm at the quartz/solution interface. Fluorescence signals were collected by an EMCCD camera (Xion 887 V, Andor Technology). The fluorescence images were recorded with an exposure time of 0.2 second. For each fluorescence image, the mean fluorescence intensity over a 37 μm × 77 μm region was calculated using Andor iQ version 8 software (Andor Technology, USA). The mean intensities were then plotted against the molar concentrations of fluorescent DNA.

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