UNIVERSITY OF CALIFORNIA, SAN DIEGO

Single Molecule Dynamics of Viral DNA Packaging Using Optical Tweezers

A dissertation written in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Physics

by

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The dissertation of Nicholas Andreas Keller is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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# Table of Contents

Signature Page ........................................................................................................................................ iii

Table of Contents ...................................................................................................................................... iv

List of Figures ........................................................................................................................................ vi

List of Tables .......................................................................................................................................... ix

List of Abbreviations ........................................................................................................................... x

Acknowledgements ............................................................................................................................. xii

Vita ......................................................................................................................................................... xiv

Abstract of Dissertation ..................................................................................................................... xvi

Chapter 1 Introduction ........................................................................................................................... 1

Chapter 2 Comments on Engineering a Dual Optical Tweezers System ........................................... 15

Chapter 3 Single-molecule measurements of Motor-driven Viral DNA Packaging in Bacteriophages Phi29, Lambda, and T4 with Optical Tweezers ........................................................ 25

Chapter 4 Non-equilibrium Dynamics and Ultraslow Relaxation of Confined DNA during Viral Packaging .................................................................................................................................................. 64

Chapter 5 Repulsive DNA-DNA Interactions Accelerate Viral DNA Packaging in Bacteriophage phi29 ....................................................................................................................................................... 85

Chapter 6 Single DNA Molecule Jamming and History-dependent Dynamics during Motor-driven Viral Packaging .................................................................................................................................................. 103

Chapter 7 Effect of DNA Screening by Cations on the Force Driving Viral DNA Ejection ............................................................................................................................................................................... 122

Chapter 8 Continuous Allosteric Regulation of a Viral Packaging Motor by a Sensor that Detects the Density and Conformation of Packaged DNA ...................................................................................... 144

Chapter 9 The Viral Packaging Motor of Bacteriophage Phi29 Exhibits High Energy Efficiency and Large Force Generation ........................................................................................................................................... 172
Chapter 10 The Viral Packaging Motors of Bacteriophages Phi29 and T4 Package Condensed DNA ........................................................................................................................................... 188

Chapter 11 Reducing Torsional Rigidity of the DNA Packaging Substrate during Motor Driven Packaging in Bacteriophage Phi29 ......................................................................................................................... 203

Chapter 12 Effect of Temperature on Viral DNA Packaging in Bacteriophage Phi29 ........ 217

Chapter 13 Evidence for an Electrostatic Mechanism of Force Generation by the Bacteriophage T4 DNA Packaging Motor ......................................................................................................................... 234
List of Figures

Figure 2.1 Optical Tweezer Schematic .............................................................. 21
Figure 2.2 Testing Resolution of Tweezers ......................................................... 22
Figure 2.3 Phi29 Step Size ............................................................................... 23
Figure 3.1 Flowcell ......................................................................................... 58
Figure 3.2 Capturing Microspheres ................................................................. 59
Figure 4.1 Motor Stall Experiments ................................................................. 77
Figure 4.2 Heterogeneity in Packaging Dynamics ........................................... 78
Figure 4.3 Motor Pausing Dynamics ................................................................. 79
Figure 5.1 Packaging Dynamics with Spermidine $^{3+}$ .................................... 96
Figure 5.2 Motor Velocity vs. Filling with Spermidine $^{3+}$ ............................ 97
Figure 5.3 Heterogeneity in Packaging Dynamics with Spermidine $^{3+}$ .......... 98
Figure 5.4 Control Packaging Experiments with Spermidine $^{3+}$ .................. 99
Figure 6.1 History Dependent Dynamics in Viral DNA Packaging ............... 114
Figure 6.2 Packaged DNA Undergoing a Jamming Transition ..................... 115
Figure 6.3 Switching the DNA Interaction During Packaging ....................... 116
Figure 6.4 Motor Velocity vs. Filling ............................................................... 117
Figure 6.5 Example Traces of Deceleration and Stalling ............................... 118
Figure 7.1 Phage Lifecycle ............................................................................ 134
Figure 7.2 Schematic of Ejection with Phi29 .................................................. 135
Figure 7.3 Measuring Un-ejected DNA Lengths with Electrophoresis ............ 136
Figure 7.4 Effect of Ionic Screening on Ejection as Revealed by Electrophoresis .... 137
Figure 7.5 Increased DNA screening Lowers Ejection Pressure ...................... 138
Figure 7.6 Partial Suppression of Ejection with High Spermine $^{4+}$ ............... 139
Figure 7.7 Comparing Ejection and Packaging Forces to a Model ............... 140
Figure 8.1 Viral Components of Phi29 ................................................................. 162
Figure 8.2 Nucleotide Exchange Experiments .................................................. 163
Figure 8.3 Analysis of Motor Pausing ............................................................... 164
Figure 8.4 Analysis of Motor Slipping .............................................................. 165
Figure 8.5 Motor Velocity Measurements ......................................................... 166
Figure 8.6 Two Different Mechanisms of Regulation ....................................... 167
Figure 8.7 Factors Influencing Motor Velocity .................................................. 168
Figure 9.1 Force Ramping Technique ............................................................... 181
Figure 9.2 High Applied Load at Low Filling .................................................... 182
Figure 9.3 Maximum Force Generated at Low Filling ....................................... 183
Figure 9.4 High Applied Load at High Filling .................................................... 184
Figure 9.5 Maximum Force Generated at High Filling ..................................... 185
Figure 10.1 Stretching Single Condensed DNA Molecules ............................ 197
Figure 10.2 Protocol for Testing Packaging with Condensed DNA ..................... 198
Figure 10.3 Packaging Rate vs. Filling with Condensed DNA ........................... 199
Figure 10.4 Phage T4 Packaging with Condensed DNA ................................... 200
Figure 11.1 Number of Nicks Packaged vs. Filling .......................................... 211
Figure 11.2 Phi29 Complexes Packaging Nicked DNA ...................................... 212
Figure 11.3 Velocity vs. Filling of Nicked vs. Non-nicked DNA ......................... 213
Figure 11.4 Phi29 Complexes Packaging Nicked DNA with High Force ............ 214
Figure 11.5 Time vs. Length of DNA packaged ............................................. 215
Figure 12.1 Diagram of Temperature Collars on Objectives ............................. 228
Figure 12.2 Velocity vs. Temperature ............................................................. 229
Figure 12.3 Velocity vs. Filling between 10 °C to 38 °C .................................. 230
Figure 12.4 Velocity vs. Force between 16 °C to 32 °C .................................... 231
Figure 13.1 Overview of T4 Packaging Motor ................................................................. 253
Figure 13.2 Force vs. Time Traces of Mutants and Wildtype ........................................... 254
Figure 13.3 Experimental vs. Computational Data ........................................................... 255
Figure 13.4 Mechanochemical Energy Landscape .......................................................... 256
List of Tables

Table 3.1 Overview of Steps ................................................................. 54
Table 3.2 PCR Parameters ........................................................................ 55
Table 3.3 PCR Mixtures ........................................................................... 56
Table 3.4 Preparation of PCR Mixtures ...................................................... 57
List of Abbreviations

DNA: Double stranded deoxyribonucleic acid

1x PBS: Phosphate-buffered saline, 10 mM Na₂HPO₄, 137 mM NaCl, and 2.7 mM KCl, pH 7.5

WLC: Worm-Like Chain Model for DNA in solution

ATP: Adenosine triphosphate

ADP: Adenosine diphosphate

Pi: orthophosphate

DNase i: Deoxyribonuclease i

EDTA: Ethylenediaminetetraacetic acid

gp16: gene product 16

gp17: gene product 17

gp10: gene product 10

gp20: gene product 20

pRNA: prohead ribonucleic acid

Spermdine³⁺: Spermdine³⁺ trihydrochloride

Spermine⁴⁺: Spermine⁴⁺ tetrahydrochloride

1x TM: 50 mM TrisHCl, 10 mM MgSO₄, pH 8.0

0.5x TMS: 25 mM TrisHCl, 5 mM MgCl₂, 50 mM NaCl, pH 7.5

s polarization: component of the electric field that is parallel to the plane of incidence

p polarization: component of the electric field that is perpendicular to the plane of incidence

nm: nanometer, 1x 10⁻⁹ m

µm: micrometer, 1x 10⁻⁶ m

phage: bacteriophage

bp: basepair
s: second

min: minutes

ASCE: additional strand catalytic ‘E’
pN: picoNewton, 1x 10^{-12} N

PCR: polymerase chain reaction

filling (fraction of genome): 100x(Length of DNA packaged/Length of Genome)

RT: room temperature, 24 C

k: Boltzmann constant, 1.38064852x 10^{-23} m^2 kg s^{-2} K^{-1}

F: Force

PEG 8000: Polyethylene glycol, average molecular weight 8000 g/mol

atm: atmospheric pressure

w/w: weight by weight

w/v: weight by volume

vs: versus
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Chapter 3, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in Methods in Molecular Biology. (N. Keller, D. E. Smith) (2016). Single-molecule measurements of motor-driven viral DNA packaging in bacteriophages phi29, lambda, and T4 with optical tweezers.

Chapter 4, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in PNAS. (Z. T. Berndsen, N. Keller, S. Grimes, P. J. Jardine, D. E. Smith) (2014). Nonequilibrium dynamics and ultraslow relaxation of confined DNA during viral packaging, Proceedings of the National Academy of Science.


Chapter 8, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in Biophysical Journal. (N. Keller, Z. T. Berndsen, D. E. Smith) (2015). Continuous Allosteric Regulation of a Viral Packaging Motor by a Sensor that Detects the Density and Conformation of Packaged DNA, Biophysical Journal.

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ABSTRACT OF THE DISSERTATION

Single Molecule Dynamics of Viral DNA Packaging Using Optical Tweezers

by

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Viral DNA packaging is a required step in the lytic cycle of many DNA viruses. A DNA polymer is packaged into a pre-formed capsid to a near crystalline density state by a powerful molecular motor that is fueled by ATP hydrolysis. To accomplish this task, the motor must generate enough force to match the resistance forces that arise from tight polymer confinement. These forces are due to entropy loss, bending energy, and electrostatic repulsion. Despite recent advances in the fields of viral DNA packaging and ejection, there are still many questions that remain unanswered. Does the packaged DNA undergo non-equilibrium dynamics? Do partly attractive DNA-DNA interactions as mediated by polyamines enhance or inhibit packaging? What affect does the conformational history of the packaged DNA have on the subsequent packaging dynamics? Which residues of the motor are responsible for generating force? And finally, is the force driving viral DNA ejection similar in magnitude to the package force? In this work, I present experimental studies that shed light on these questions. The packaging of single DNA molecules into single empty pro-capsids was
measured using a custom-built dual optical tweezers system. Using the bacteriophage phi29 and T4 systems, I and my research collaborators pioneered new techniques in optical tweezers for investigating the dynamics of DNA packaging and force generation of a viral packaging motor. Using these techniques, we discovered many new findings in the areas of polymer confinement, non-equilibrium dynamics, DNA condensation, and enzyme kinetics.
Chapter 1

Introduction
Viral DNA Packaging and Ejection in Bacteriophages

Bacteriophages ("phages") are viruses that infect bacteria, are among the most numerous organisms on earth, and are found in almost all places on earth. The size of a phage is on the order of ~10-100 nm and the geometric shapes of phages are observed to vary from cylinders to spheres [1]. The lifecycle of a phage begins when a fully packaged phage attaches itself to the membrane of the host bacterial cell and subsequently injects its DNA ("viral DNA ejection") into the cell cytoplasm [2]. The cell machinery produces the viral proteins that then assemble into prohead intermediates. These prohead intermediates then package the viral DNA into their empty proheads ("viral DNA packaging"). After packaging, the cell membrane lyases leading to cell death releasing the fully packaged phages. These fully packaged phages repeat the ejection/packaging cycle by infecting nearby bacteria cells.

A critical step in the life cycle is DNA packaging. Many dsDNA bacteriophages and some dsDNA eukaryotic viruses such as herpesvirus and adenovirus carry out this process. A DNA molecule is packaged into a pre-formed protein shell called a prohead or procapsid by a molecular motor to a near crystalline density state. To achieve this, the motor, which is fueled by the energy released from ATP hydrolysis, must overcome the forces that arise from tight polymer confinement, which are due to entropy loss, bending energy, and electrostatic repulsion. This remarkable process occurs in only ~5 min at room temperature (~20 °C) and exhibits an in-vivo packaging efficiency that approaches 100% [3]. The ejection process is an equally important step during viral infection. During ejection, the packaged DNA is released out of the prohead through the portal channel and into the cell cytoplasm. Ejection is driven by the large change in free energy between a DNA molecule in solution and the fully packaged DNA [4].

Robust bulk, in-vitro packaging assays were developed and optimized by my collaborators [5-7]. Bulk packaging is initiated by mixing purified samples of proheads, motor
protein, DNA, and ATP. After waiting 15 minutes to allow packaging to complete, DNase I is added to digest any remaining unpackaged DNA. Since DNase I cannot permeate through the capsid walls, the packaged DNA is protected during this digestion. A mixture of proteinase K and EDTA is added to digest and inhibit DNase I, and digest the proheads. Digesting the proheads releases the packaged DNA. Standard gel electrophoresis is applied to measure the amount and length of DNA packaged. The in-vitro bulk packaging protocol for T4 is nearly identical[8].

Viral DNA ejection has been measured in many dsDNA viruses such as Lambda, T5, SPP1, and herpesvirus [9-12]. Ejection, in the normal biological process, occurs through a tail tube, which is assembled onto the portal channel after packaging is completed and the motor dissociates. The standard mechanism for ejection is a receptor protein, which binds to the tip of the tail and triggers the release of the packaged DNA. Since work was required to package the DNA into the prohead, ejection is driven at least in part by the change in free energy between the packaged DNA and free DNA in solution. An osmotic pressure technique was developed by the Gelbart and Knobler laboratory at UCLA to estimate the amount of pressure that is required to drive ejection [9]. We have very recently developed an efficient ejection protocol for phi29. Our ejection results are discussed in Chapter 7.

**Viral Components of the Bacteriophages**

The force generating molecular motor of a dsDNA virus is attached at a unique vertex of the viral prohead. Situated at the vertex of the prohead is a portal protein ring that serves as the binding site for the portion of the motor responsible for generating force and hydrolyzing ATP, and the DNA is translocated through this portal channel [1,13]. In this work, I conducted single molecule measurements of this process with phages phi29 and T4.

Structural and biochemical studies of phi29 have shown that the molecular motor is composed of a homomeric ring of gene product 16 (gp16), an oligomeric ring of prohead RNA
(pRNA), and a dodecameric connector protein ring (gp10) [3,14,15]. Each ring is stacked on top of one another in a coaxial geometry and during packaging the DNA pierces through the center of each ring and into the prohead. The components of the motor necessary for ATP hydrolysis and force generation are pRNA and gp16. Both single molecule and cryo-electron microscopy data strongly suggest that the motor is a ring composed of five or six gp16 monomers [16-18]. For my in-vitro DNA packaging studies, I received purified samples of gp16 and proheads, which include the pRNA ring, from our collaborators, Professors Paul J. Jardine and Shelley Grimes at the University of Minnesota.

In phage T4, structural and biochemical studies have shown that gp20 forms a dodecameric portal protein ring and DNA translocation and endonuclease activities reside in a terminase complex, comprised of a ring of large (gp17) and small terminase (gp16) subunits [19]. The large subunit is responsible for ATP hydrolysis, force generation, and nuclease activity, whereas the small subunit on its own has no ATPase or nuclease activity; it is thought to be involved in recognition of the DNA substrate in vivo [20]. In fact, if gp16 is included during packaging in the current implementation of the in vitro packaging assay, the efficiency of packaging significantly drops [21]. Thus the T4 packaging studies in this work did not use gp16. We received purified gp17 monomers and purified T4 proheads from our collaborator, Professor Venigalla Rao at Catholic University of America.

DNA Condensation

Above a critical concentration, a cation with a charge of +3 or greater will cause DNA in solution to undergo a structural transition from an expanded, worm-like chain to a tightly wound, toroidal spool, referred to as “DNA condensation” [22,23]. According to dynamical simulations and analytical theories, the DNA strands are predicted to be arranged together as an hexagonal lattice, which interestingly is the same predicted cross-sectional arrangement of packaged DNA in the inverse spool model [24,25]. Polyamines are cations that contain one or
more positively charged amino groups (-NH$_2$). Spermine$^{4+}$ and Spermidine$^{3+}$ are polyamines present in the cell at millimolar concentrations, which is conceivably enough to condense DNA [26]. Their role on DNA condensation in vitro has been extensively studied using both single molecule and bulk methods. Electron microscopy measurements have shown that DNA molecules in solution will condense into toroidal spools with a diameter ranging between 50 and 300 nm [22]. Bulk studies have shown that the relaxation time of these structures ranges between 10 minutes to many hours [27].

**Modeling the Viral DNA Packaging Process**

The viral DNA packaging and ejection process has been modeled theoretically by several groups [4,28-30]. To predict the packaging energetics and DNA conformation, theorists either develop analytical theories or carry out molecular dynamical simulations. In analytical theory models, packaging is assumed to occur under quasi-static conditions, which implies that the packaged DNA rapidly relaxes to a free energy minimum state. In molecular dynamical simulations, the packaged DNA is allowed to undergo non-equilibrium (dissipative) dynamics. In both approaches, theorists find that the forces resisting polymer confinement are due to electrostatic repulsion, entropy loss, and bending energy. The relative importance of each of these factors is still debated, although all models predict that the internal force rises with increasing DNA density.

The Philips laboratory at Caltech and the Gelbart/Knobler laboratory at UCLA have both developed a continuum-mechanical model that predicts the energetics and forces of packaging and ejection (4, 25). They employ an empirical DNA-DNA interaction potential based on measurements of osmotic-pressure induced DNA condensation by Rau and Parsegian [31]. Both groups find that the force reaches a final value that ranges between 30 and 60 pN at 100% prohead filling [4,32]. The largest contributor to the internal force at low filling is the force due to bending energy and the largest contributor to the internal force at high filling is
due to electrostatic repulsion. These models do not explicitly include entropy change associated with conformational change, but the empirical interaction potential includes entropic effects relating to the case of condensation of short DNA segments into hexagonally packed arrays, studied by Rau and Parsegian, including hydration effects related to changes in the arrangement of water molecules around the DNA. During the early stages of packaging, the DNA wraps around the outer perimeter of the prohead and proceeds to spiral toward the center of the prohead as more DNA is packaged. This predicted DNA trajectory is called the inverse-spool model because the process is opposite to how a ball of yarn is wound up.

The Harvey laboratory has conducted multiple molecular dynamical simulations on viral DNA packaging [28,33-36]. Using the physical geometry of phi29, they predict the force to rise to 30 pN and the trajectory of the DNA to proceed in an inverse-spool way consistent with the results from continuum mechanical models [4,37]. However, they find that the change in entropy of the DNA is the largest contributor to the total energy.

All theoretical studies found that partly attractive DNA-DNA interactions enhance packaging by reducing the forces that resist confinement [4,35]. Various structures of the final packaged DNA are predicted, including spool-like and folded toroid shapes, generally being more ordered than in the case with repulsive interactions [4,28].

These theoretical studies are important for my work on packaging. We compare our experimental results to theoretical predictions in order to gain a better understanding of the forces and energetics that drive viral DNA packaging and ejection.

**Manipulation of Single DNA molecules with Optical Tweezers**

The construction of an optical tweezers system requires detailed component design and careful procedures for aligning opto-mechanical instruments. During my time as a graduate student, I have successfully engineered three optical tweezer systems and wrote data
acquisition and device interfacing software for manipulating the optical tweezers. A more detailed discussion of optical trap design and construction is discussed in Chapter 3.

The protocols that I developed for conducting packaging measurements with optical tweezers were based on pre-existing single molecule protocols that were developed by previous lab members and bulk packaging protocols that were developed by my collaborators. I was very fortunate to have worked with Professor Venigalla Rao of Catholic University and Professors Shelley Grimes and Paul Jardine of University of Minnesota. Throughout my studies, they continuously offered helpful advice and gave me valuable targeted feedback, and provided me with purified motor protein and proheads. Without their help, I would not have been able to conduct any packaging measurements. The protocol for preparing complexes for single molecule measurements with optical tweezers is very similar to the bulk packaging protocol but with many minor modifications. A more detailed discussion of the methods developed by me and my collaborators is discussed in Chapter 4.

In a typical measurement, a single DNA molecule is attached to one trapped microsphere and a stalled prohead-motor complex is attached to another trapped microsphere. Bringing the two microspheres in close contact initiates packaging because the motor will sometimes grab the free end of the DNA molecule and begin to reel it into the capsid. We control the distance between the two microspheres and measure the force exerted on the microspheres. Typical quantities we compute are packaging rate as a function of DNA packaged at a fixed force or packaging rate as a function of increasing force.

The manipulation of single prohead motor complexes with optical tweezers reveals much more information on the packaging dynamics than with conventional bulk techniques. In a bulk packaging experiment, only the average amount of DNA packaged after a defined waiting time of 5 minutes is measured and little is known about the instantaneous motor velocity or distribution of DNA lengths among individual prohead-motor complexes. Since we
measure single DNA molecules being packaged into single empty proheads, we determine both the average and distribution of packaged DNA lengths. We also perform our measurements in real time so that we can compute time dependent quantities such as the packaging rate and resolve heterogeneous events such as pauses and slips, which bulk techniques cannot do. For example, a conventional bulk packaging experiment predicts that phage T4 packages its 170 kb genome genome ~6-fold faster than phage phi29 (19.3 kb genome) because both complete the packaging process in the same amount of time. However, measurements with optical tweezers reveal that their dynamics are widely different [38,39]. Phage T4 exhibits a broader distribution of packaging velocities than phage phi29.

Application of forces and performing rapid buffer exchanges on the motor are also extremely useful tools that are not available with bulk techniques. We can measure how an active, individual prohead-motor complex responds to different forces. These measurements reveal information on the mechano-chemical cycle of the motor during the ATP hydrolysis reaction. In preliminary work with phage lambda we have also observed discrete spatial steps of the motor as it is packaging. Rapid buffer exchanges are also performed to measure how a different ionic condition changes the packaging dynamics. For example, we observed history dependent dynamics and single molecule jamming in viral DNA packaging by conducting rapid solution exchanges between a high Spermine$^{4+}$ solution and the standard packaging solution. These results are discussed in Chapter 6.

**Brief Overview of Chapters**

Viral DNA packaging is an interesting problem from the points of view of both soft matter physics and molecular biophysics/biochemistry. My research as a graduate student has investigated both aspects of packaging. Phi29 was used as a model system to investigate the dynamics of DNA under tight confinement because, among the phages that can be re-assembled from purified proteins, it exhibits the least heterogeneity in motor velocity, exhibits
the highest packaging efficiency, and the length of its genome is within the working range of
the optical tweezers allowing me to accurately measure packaging at all prohead filling levels.
For identifying which residues of the motor protein are responsible for force generation, I used
the T4 system because the x-ray crystal structure of the motor protein sub-unit (gp17) is
known and our collaborator supplied us with mutant proteins that had changes in specific
amino acid residues[40]. I also conducted additional studies on the wildtype motors of T4 and
phi29.

Chapter 2 discusses the single molecule packaging protocols I developed for T4 and
phi29, and the protocols for making the DNA packaging substrates using long range PCR.

Chapter 3 is a summary of the protocols that I developed for building a dual optical
tweezer system having improved stability compared with the previous one used in our lab.

Chapter 4 addresses the question of whether packaging undergoes non-equilibrium
dynamics. We developed a new solution exchange technique to directly show that packaging
exhibits non-equilibrium dynamics. In addition, we show that motor pausing is directly
associated with the conformational state of the DNA.

Chapter 5 addresses the question of whether packaging with a concentration of
polyamines that induces DNA condensation inhibits or enhances packaging. Theoretical
studies predict that a partly attractive DNA-DNA interaction enhances packaging because the
forces resisting confinement are reduced [4,28]. We however find that packaging with an
attractive interaction causes heterogeneous dynamics. Acceleration is observed in 25% of
complexes, but ~75% exhibit slowing and stalling, suggesting that attractive interactions
promote non-equilibrium DNA conformations with very long relaxation times that impede
motor translocation.

Following the results of Chapter 4 and Chapter 5, Chapter 6 further investigates the
inhibition to packaging that is observed with partly attractive DNA-DNA interactions. We
provide evidence suggesting that the stalling is due to the packaged DNA undergoing a jamming transition akin to that observed with granular and colloidal systems in condensed matter physics. We show that packaging exhibits many of the hallmarks of a jamming transition such as force induced un-jamming, history dependent dynamics, and un-jamming by switching the DNA-DNA interaction from net attractive to net repulsive.

In Chapter 7, I discuss viral DNA ejection experiments with phi29. We carried out ejection measurements under different ionic conditions. Consistent with theoretical predictions, we find that the ejection force decreases with increased DNA screening [4,28,32].

In Chapter 8, we identified an allosteric interaction in phage phi29 between the packaged DNA and the motor. The motor senses the conformational state of the packaged DNA and reduces the motor speed by reducing the ATP binding rate in order to reduce non-equilibrium affects that inhibit packaging. Using motor slipping as an internal force sensor, we find that the internal force begins to sharply increase at 75% filling and reaches a final value of only ~22 pN at 100% filling, which is 80% smaller than the previously reported estimate, and consistent with some early theoretical predictions [4].

In Chapter 9, we use high applied forces and the revised internal force versus filling relationship to put a revised lower-bound on the maximum force the phi29 motor can generate. We find that the motor can generate at least 89 pN of force indicating that at least 60% of the chemical energy released by ATP is converted into mechanical work.

In Chapter 10, we show that T4 and phi29 are able to package externally-condensed DNA. While many structural and theoretical studies have investigated the effect of a partly attractive DNA-DNA interaction on the conformation of the packaged DNA, no study to date has directly shown that viral motors are capable of packaging condensed DNA segments [4,28,41]. Since the minimum diameter of a toroidal spool is ~50 nm and the average diameter
of the portal channel is ~0.3 nm, the motor must de-condense a toroidal spool by forcing it through its portal channel.

In Chapter 11, we measured phi29 packaging with a DNA substrate that has been heavily nicked. By packaging with a nicked substrate, we address the extent to which torsional and bending rigidity plays an important role in governing the packaging rate and forces resisting packaging. We find that packaging with a heavily nicked substrate causes ~40% of complexes to stall before reaching 100% filling.

In Chapter 12, we investigated whether packaging at altered temperatures affects the packaging rate, internal force, and motor operation. We find that temperature does not largely influence the packaging dynamics.

In Chapter 13, we compared the packaging dynamics between the T4 wildtype motor and three T4 mutant motors. Charged residues located on the motor that are predicted to be critical for force generation were replaced with oppositely charged residues. We carried out packaging measurements with these mutants at different forces and also performed molecular dynamics simulations. The simulation data agree with the experimental data and lends support to a recently proposed model for T4 motor operation [40].
References


Chapter 2

Comments on Engineering a Dual Optical Tweezers System
Abstract

A linearly polarized fiber laser operating at a near infrared wavelength (1064 nm) is split into its orthogonal polarizations, which form the two optical traps. One beam bounces off a fixed mirror and remains spatially fixed while the other beam bounces off a piezo-controlled rotating mirror and forms the movable trap. The beams are again collected and focused with a high numerical aperture water immersion objective onto a custom built microfluidic flow-chamber, which is where optical trapping occurs. In one method, bringing the prohead-motor microsphere into close proximity with the DNA microsphere initiates packaging and packaging is then tracked and manipulated in real time by simultaneously monitoring the force exerted on the microsphere and adjusting the distance between the microspheres. Software written in LabVIEW and C are used to simultaneously acquire data and send control signals to opto-mechanical devices in all optical tweezers experiments. In-house MATLAB programs are used to analyze the data. We built a high resolution laser tweezer system that implemented differential detection between the two optical traps and used improved components and design to improve the thermal and mechanical stability of the system. This system is able to resolve 5 bp steps in a 1 Hz bandwidth window. In addition, we are able to resolve the 10 bp step size of the phi29 packaging motor. We are currently conducting measurements to try to detect the motor step size in other packaging systems such as lambda and T4.

Design, alignment, and construction of an optical tweezers

A linearly polarized laser with an initial laser beam diameter of ~1.6 mm operating at the near infrared wavelength is first expanded by a factor of 2 with a beam telescope (Figure 1). A beam telescope consists of two lenses with the distance between the lenses equal to the sum of their focal lengths. The beam is split into its “s” and “p” polarizations with a polarizing beam splitter (“PBS”). The “p” beam bounces off a fixed mirror and forms the fixed trap and the “s” beam bounces off a piezo-controlled mirror and forms the movable trap. The piezo-
controlled mirror is a DC-voltage controlled device that is stable to within ~1 nrad on a 0.1-100 Hz frequency scale and can scan at a maximum rate of 400 Hz. An acoustical optical deflector (AOD) can also be used as a beam deflector. The AOD can scan at much faster rates (~1 MHz). However, a drawback of the AOD is that the power of the deflection beam changes with deflection angle, by up to ~10% at the largest angles used, which implies that the trapping force is not constant at different relative trapped microsphere distances. The beams are collected by a PBS and expanded again by a factor of ~3.2 such that the diameter of the beam is slightly larger than the diameter of the back aperture of the microscope objective (~9 mm). The objective focuses the beam inside a custom built flow chamber where packaging is measured. In order for a rotation from the movable mirror to be converted into a linear translation in the optical trapping plane, two conditions must be satisfied. First, the distance between the movable mirror and the first lens of the second telescope must equal the focal length of that lens (Figure 1). Second, the distance between the back focal plane of the trapping objective and second lens of the second telescope must equal the focal length of that lens. The beam is then collected by an identical objective and re-collimated. A lens is used to image the back focal plane of the second objective onto a position sensing detector (“PSD”).

The force on the trapped microsphere, which is also equal to the DNA tension, is proportional to the PSD signal. To reduce spherical aberration, we use achromatic lenses that have an anti-reflective coating on the lens surface.

The main factor that degrades the resolution of the force signal, besides the unavoidable Brownian motion of the microspheres, is environmental noise [1]. Variations in temperature between different sections of the system cause fluctuations in the refractive index of air and cause metallic, opto-mechanical components such as lens holders and mirror posts to thermally expand and contract. These effects cause the pointing stability of the trapping beam to fluctuate. Local air currents cause convection, which is a form of heat transfer,
leading to further additional laser pointing instability. To circumvent these issues, the optical
tweezers should be extremely well insulated from air currents and thermal fluctuations. Many
labs such as the Block laboratory at Stanford and the Bustamante laboratory at UC Berkeley
operate their optical tweezer system in a temperature controlled, vibration isolated, and sound
proof room, but this is very expensive [1,2]. The first tweezer I built was isolated by incasing
the system in plexiglass. The second optical tweezer system I built with a fellow graduate
student, Damian del Toro, was further insulated by encasing the laser path with anodized
aluminum tubes. These precautions are adequate for most types of measurements we are
interested in.

The position sensing detector simultaneously measures the horizontal force acting on
the trapped microsphere and the laser intensity. We typically only measure the force that is
exerted on the fixed trap. However in addition to measuring the force, we also measure the
Brownian motion of both trapped microspheres [1,3,4]. Since a molecular motor typically
operates at frequencies below 50 Hz, we remove the high frequency portion of the signal by
passing it through an electronic low pass filter with a cut-off frequency of 300 Hz. We sample
at frequencies larger than 1 kHz so that we are not aliasing.

Reference 1 has experimentally shown that if both traps are simultaneously measured
and the signals of each trap are subtracted, then the signal to noise ratio increases. Motion that
is symmetric, which includes the Brownian motion of each microsphere and the environmental
noise that is picked up by both of the traps in sections of the system where they travel along
the same optical path, is subtracted. Motion that is anti-symmetric, which includes any force
that reduces the distance between the microspheres, is conserved [1]. This is analogous to how
an instrumentation difference operational amplifier works. The signal that is common to both
inputs is rejected while the anti-symmetric portion of the signal is conserved. We built a high
resolution optical tweezers that uses differential detection. We isolated the optical tweezers
system from environmental noise by covering the laser path with anodized aluminum tubes and incasing the entire optical system with plexiglass.

According to the worm-like chain (WLC) model, the DNA extension ($x$) varies with applied force ($F$) as, $x/L_{DNA} = 1 - \sqrt{4FP/kBT} + F/S$, where $P$ is the persistence length, $S$ is the stretch modulus, and $L_{DNA}$ is the length of the DNA molecule [5]. If the optical tweezer system is aligned and calibrated properly, the DNA extension is equal to the distance between the traps minus the sum of the displacements of each microsphere from the center of their traps. In equation form, the end-to-end distance of a stretched DNA molecule equals, $x = \beta(V_o - V) - \gamma\alpha(V_{PSD1})$ where $\beta$ [$nm/V$], $\alpha$ [$pN/V$], and $\gamma$ [$nm/pN$] are calibration parameters (units of each parameter are in square parenthesis), $V$ is the driving piezo voltage which controls the distance between the traps, and $V_{PSD1}$ is the PSD sensor voltage of the fixed optical trap. The sensor voltage of the PSD is proportional to the force that the trap exerts on the trapped microsphere [6]. Equating these two formulas gives the DNA length as a function of piezo driving voltage and PSD sensor voltage.

Increasing the distance between the microspheres increases both the tension and the length of the DNA molecule. For example, under our current set of calibration parameters, increasing the relative distance between the trapped microspheres by 5 nm at a DNA tension of 20 pN increases the DNA length by 3.4 nm. We increased the distance between the microspheres with a fixed step size every second to estimate the resolution of our newly built high resolution system. We can detect 5 basepair (1.7 nm) steps at forces larger than 10 pN (Figure 2) in a 1 second time window.

We also tested the capabilities of our system by conducting measurements with phi29. It has been shown that the step size of phi29 during packaging is 10 bp (actually consisting of a fast burst of four 2.5 bp sub-steps that can be observed under high applied loads) [7]. We also observe 10 bp steps in some our traces. An example trace is shown in Figure 3.
Data acquisition and device interfacing

The two types of measurement modes that we use during packaging experiments are force clamp and position clamp mode. In force clamp mode, the force is held constant by increasing/decreasing the trap distance if the DNA tension decreases/increases. This force feedback program is written in LabVIEW. It uses a software timed loop that runs at 1 or 2 kHz. To minimize loop jitter, I regulate the loop speed by using a dynamic link library, which I wrote using Microsoft Visual C++, that prints out a time stamp of the computer CPU clock. To reduce the impact of noise that results from the trapped microsphere’s Brownian motion and to avoid aliasing of higher frequency noise, an electronic low pass filter (resistor capacitor circuit or Bessel filter circuit) is used with a roll-off frequency of 300 Hz. In position clamp mode, the position of the two traps is held constant causing the force to continuously rise during a packaging measurement. Much higher resolution is achieved with this measurement mode because both traps are held fixed during packaging, avoiding inaccuracy of feedback controlled beam steering, and the rise in force is entirely due to packaging. The data acquisition rate is typically 100 kHz, which is much higher than the corner frequency of a trapped microsphere (~5 kHz) [1]. For the experiments that measured the step size of the motor, I wrote a variable force clamp program that keeps the force within a pre-set fixed range and samples at 100 kHz.

Data analysis

All the data were analyzed using custom MATLAB programs. Typical quantities that I compute are rate of DNA packaged, length of DNA stretched versus applied force, and the power spectrum of the trapping laser versus frequency. To speed up computational time, I parallelized portions of my code by writing them in C and using OpenMP directives.
Figure 2.1 Optical Tweezers Schematic. The dual optical tweezer system measures single DNA molecules being packaged into single viral proheads in real time. For clarity, the path of the light source that is used for imaging the trapped microspheres is not shown.
Figure 2.2 Testing Resolution of Tweezers. The change in DNA tether length was measured by increasing the distance between the traps by 2.5 nm every second. The applied tension during this measurement was ~30 pN. The tether length in (A) is from a single trap and the tether length in (B) is computed by subtracting the tether length of the fixed trap from the movable trap. The change in DNA tether length in (B) is 5 bp as revealed by the pairwise distribution graph in (C). According to the WLC model, the predicted step size is also 5 bp.
Figure 2.3 Phi29 Step Size. The phi29 motor packages DNA with a step size of 10 bp which is consistent with a previous high resolution single molecule study (7).
References


Chapter 3

Single-molecule Measurements of Motor-driven Viral DNA Packaging in Bacteriophages Phi29, Lambda, and T4 with Optical Tweezers
Abstract

Viral DNA packaging is a required step in the assembly of many dsDNA viruses. A molecular motor fueled by ATP hydrolysis packages the viral genome to near crystalline density inside a pre-formed prohead shell in ~5 minutes at room temperature. We describe procedures for measuring the packaging of single DNA molecules into single viral proheads with optical tweezers. Three viral packaging systems are described in detail: bacteriophages phi29 (φ29), lambda (λ), and T4. Two different approaches are described: 1. With φ29 and T4, prohead-motor complexes can be pre-assembled in bulk and packaging can be initiated in the optical tweezers by “feeding” a single DNA molecule to one of the complexes; 2. With φ29 and λ, packaging can be initiated in bulk then stalled, and a single prohead-motor-DNA complex can then be captured with optical tweezers and restarted. In both cases, the prohead is ultimately attached to one trapped microsphere and the end of the DNA being packaged is attached to a second trapped microsphere such that packaging of the DNA pulls the two microspheres together and the rate of packaging and force generated by the motor is directly measured in real time. These protocols allow for the effect of many experimental parameters on packaging dynamics to be studied such as temperature, ATP concentration, ionic conditions, structural changes to the DNA substrate, and mutations in the motor proteins. Procedures for capturing microspheres with the optical traps and different measurement modes are also described.

Introduction

Overview: DNA packaging is a fundamental process in the lytic cycle of many dsDNA viruses. An ATP-powered molecular motor translocates a single DNA molecule into a single pre-formed prohead to near crystalline density [1-4]. The motor must overcome the forces resisting DNA confinement which arise due to electrostatic self-repulsion of DNA strands, entropy loss, and DNA bending energy [5-10]. In vitro bulk packaging assays using defined
sets of purified viral proheads, motor proteins, and DNA have been developed for bacteriophages λ, φ29, and T4 [11-13]. The integration of these methods with optical tweezers techniques [14-16] has allowed for direct manipulation and measurement of DNA packaging by single prohead-motor-DNA complexes [3,17-20].

In our lab we have developed techniques for measuring DNA packaging in phages λ, φ29, and T4 using optical tweezers. We find that these motors package DNA at rates ranging from 100-2000 bp/s in saturating ATP at room temperature, generate forces >50 pN, and exhibit a trend of monotonically decreasing average motor velocity with increasing prohead filling or with increasing applied force [17-19,21]. These methods have been applied to study the function and mechanism of the motor [22-30] and the nature of the forces resisting DNA confinement [28,31-33].

In this chapter we describe procedures for measuring single DNA molecules being packaged into proheads with optical tweezers for phages φ29, T4, and λ. While the detailed steps of the protocols between each system differ, many steps of the basic procedures are the same (Table 1.1). Biotin end-labeled DNA substrates are prepared by PCR. To measure phage packaging in the tweezers we utilize two basic different approaches: in situ initiation or restart of stalled complexes. All procedures are described except for the preparation of proheads and motor proteins, as these are prepared in the laboratories of our collaborators and have already been described in previous publications [11,13,18].

For the in situ method, prohead-motor complexes are first formed and stabilized, by addition of a non-hydrolyzable ATP analog (γ-S-ATP), in a bulk reaction and then attached to anti-prohead coated microspheres. The DNA, which is biotin end-labeled, is attached to streptavidin microspheres. Inside the flow chamber of the tweezers instrument, which contains a packaging buffer with ATP, one of each of these microspheres is captured in each of the two optical traps. By bringing both microspheres close together, the end of a single DNA substrate
from the DNA coated microsphere is brought into contact with the motor of a single prohead-motor complex on the microsphere in the opposing trap, causing packaging to initiate. We have demonstrated that this in situ protocol works for phages φ29 and T4 [19,21,29,31,33]. We note that a different in situ protocol was initially developed for λ phage in which motor-DNA complexes are formed, attached to microspheres, and packaging is initiated by bringing one of these microspheres into contact with a prohead-coated microsphere. This protocol has been described previously in Ref. [18] and is not discussed further here because we have found that it is much less efficient than the stall-restart protocol described below. By "efficiency" of the assays we mean success rate for recording packaging events. In practice it can be highly variable and difficult to quantify, but is roughly proportional to the relative number of packaging events that initiate with the correct DNA tether length measured per pair of microspheres tested. Efficiencies we consider workable range anywhere from one event per several pairs of beads tested down to one event per several hundreds of beads tested.

For the stall-restart method, prohead-motor-DNA complexes are assembled and packaged in bulk for a short while (typically enough time for ~20% of the genome to be packaged) before the process is stalled by addition of a non-hydrolyzable ATP analog (γ-S-ATP). These stalled complexes are then attached to streptavidin microspheres via the biotinylated unpackaged DNA end. Packaging is measured inside an experimental flow chamber in the optical tweezers instrument by bringing a trapped anti-prohead microsphere into contact with the complex such that the prohead binds to the anti-prohead microsphere. Packaging restarts when this tethered prohead-motor-DNA complex is exposed to ATP. In the optical tweezers measurements, a data acquisition and instrument control program either maintains a constant DNA tension (referred to as “force-clamp mode”) while controlling the separation between the traps or leaves a constant distance between the traps while measuring applied force (referred to as “fixed trap separation mode”).
In our dual-trap optical tweezers systems [34-36], the force signal of one trap can be affected by the second trap when the relative distance between the two traps is small, which we refer to as “cross-talk”. In our system, effects of cross-talk occur when the distance is less than ~1 kbp, in terms of stretched DNA length. This causes the force measurement to fluctuate ~0.1-10 pN depending on separation [35]. To avoid having to correct for this effect, we prefer to keep a ~1 kbp separation between the traps during measurements and thus we design our DNA substrates to be at least 1 kbp longer than needed for packaging in a particular experiment.

**Phage φ29 packaging:** Bacteriophage φ29 is a virus that infects *Bacillus subtilis* [37]. The dimensions of its prolate icosahedral prohead are 54 x 48 nm and the length of the genome is 19.3 kbp (6.6 μm). The viral genome is somewhat unusual in that it has a protein, gp3 (gene product 3), covalently attached to its 5' ends (some other viruses including adenoviruses also have such terminal proteins). Gp3 acts as a primer for φ29 polymerase during DNA replication and it also has been shown to significantly increase the efficiency of packaging [11,38]. The motor of φ29 is composed of three ring structures, which are the head-tail connector (gp10), prohead RNA (pRNA), and gp16 (the ATPase). They are sandwiched together and attached to a unique portal vertex of the prohead which is where the DNA enters [37]. To assemble motor complexes, we mix purified gp16 monomers and purified proheads (which already have the connector and pRNA attached).

For φ29, we perform DNA packaging measurements using both the *in situ initiation* and *stall-restart* methods where the particular method used depends on the desired experimental conditions. For the *in-situ* method, proheads, gp16, and γ-S-ATP are mixed together in bulk to form empty prohead-motor complexes. For the *stall-restart* method, bulk packaging is initiated by first combining proheads, gp16, gp3-DNA and ATP. After incubating the mixture for ~20 s, which causes ~20% of the genome to be packaged, γ-s-ATP is added to
stall the packaging reaction and generate stable, partially-filled prohead-motor-DNA complexes. In the in-situ method the packaging dynamics can be tracked from shortly after initiation to completion because packaging is initiated when the DNA is tethered in the optical tweezers.

With the stall-restart method, the packaging measurement begins with ~20% of the genome already packaged. An advantage of the in-situ method is that it enables us to measure the early filling dynamics. However, a drawback with the in-situ method is that we find the initiation efficiency can decrease significantly in conditions where temperature, ATP concentration, or ionic condition is varied from the standard conditions. This is not an issue in the stall-restart method if the stalled complexes are prepared in bulk under the standard conditions; the conditions can then be changed in the flow chamber during the optical tweezers measurement.

Unlike the situation in bulk packaging where gp3-DNA is needed for efficient packaging, we have found that non-gp3 DNA of arbitrary sequence can be used to initiate packaging efficiently in the in situ method. We often use a 25,339 bp DNA substrate generated by PCR from λ phage DNA, where the 5’ end of one primer is labeled with biotin and the 5’ end of the other primer is labeled with digoxigenin [39]. The biotin is used to tether the DNA to streptavidin microspheres in the packaging measurements while both the biotin and digoxigenin labels are used in control measurements for confirming proper DNA amplification and labeling (by tethering the DNA between streptavidin and anti-digoxigenin microspheres). In the stall-restart method we use the native φ29 gp3-DNA as the DNA packaging substrate because bulk packaging is very inefficient without gp3 [11]. However, as we mentioned earlier, since cross-talk perturbs measurements of the final ~1 kbp of packaging with the φ29 genome in our optical tweezers system, we usually prepare a longer hybrid φ29 construct in which the length is increased by ~8 kbp by ligating a biotinylated 12.2 kbp segment of DNA
to a 15 kbp section of the φ29 phage DNA, which still contains the gp3 protein at one of its ends. Since ligation is somewhat inefficient, the yield of this DNA construct is lower than the 25.3 kbp PCR generated DNA used with the in situ method and the efficiency of obtaining packaging events is also lower.

**Phage T4 packaging:** Bacteriophage T4 infects *E. coli*. The terminase complex, which functions both as a DNA translocating motor and endonuclease that can excise a unit-length genome for packaging from a concatameric DNA substrate, is composed, *in vivo*, of multiple large terminase subunits (gp17) and small terminase subunits (gp16) [40]. The terminase complex binds to the portal ring (composed of multiple gp20 subunits) which is situated at a unique vertex of the empty prohead. The dimensions of the filled capsid are approximately 86 x 120 nm and the length of the genome is ~171 kbp. For this phage, we have developed a protocol for measuring DNA packaging experiments in the tweezers using the *in situ* approach, which works well, but have not tested a stall-restart approach (which would be somewhat difficult due to the high DNA translocation rate). We and our collaborator (Dr. Venigalla Rao) have also developed an improved hybrid method in which a short 120 bp DNA segments are included during bulk assembly of prohead-motor complexes. The inclusion of these segments appears to stabilize the prohead-motor complex formed in the presence of γ-S-ATP; this DNA, which is negligible in length compared to the T4 genome, may be packaged when the complex is exposed to ATP. The T4 motor can package multiple DNAs sequentially and we find that inclusion of this short DNA promotes efficient *in situ* initiation of packaging with a longer DNA in the tweezers assay [29]. Proheads, gp17, γ-s-ATP, and 120 bp DNA are mixed together to promote the formation of prohead-motor complexes. While gp16 is part of the terminase complex *in vivo*, it does not contain the ATPase activity responsible for DNA translocation and we have found that it is not needed for *in vitro* packaging in the tweezers assay [19]. For the tweezers measurements we use the same 25,339 bp DNA substrate as in the
φ29 protocol because, like φ29, we have found that it is not necessary to use T4 DNA (the whole T4 genome length is also too long to manipulate in our tweezers).

**Optical tweezers measurements:** Typically, optical tweezers use a laser that operates at the near IR wavelength (to reduce damage to the biological molecules), runs at 1 W, and is linearly polarized. In a dual-trap system, a single beam is split into two orthogonally polarized beams where one beam reflects off a fixed mirror (this beam corresponds to the fixed trap) and the other beam is steered by a mirror or acousto-optic deflector (this beam corresponds to the movable trap, which controls the separation between the two traps) [41]. The beams are then collected and focused into a custom built flow chamber. Within this flow chamber, the user manually captures virus coated microspheres and manipulates them so that the packaging of a single DNA substrate by a single prohead-motor complex can be measured. The force exerted by the molecular motor on a trapped microsphere is determined by using a position sensing detector (PSD) to measure the angular deflection of the trapping laser, which is converted into force using an appropriate calibration method [34,35].

The flow chamber we use in our experiments, based on a design by Steven Smith [42], contains three nearly isolated chambers (Fig. 1.1). The volume of each chamber is ~100 μl and the top and bottom chambers are connected to the central chamber by thin glass capillary tubes with an inner diameter of ~25 μm. Packaging experiments are performed in the central channel, through which we gently flow a packaging buffer. Two different sets of microspheres are then injected into the top and bottom chambers. The capillary tubes then transfer some of the microspheres from these chambers into the central chamber where they are captured and manipulated for the experiment.

As mentioned earlier, we conduct packaging measurements with either a force-clamp or fixed trap separation mode data acquisition and instrument control program. These programs are written in LabVIEW and C. In force-clamp mode, the tension of the DNA is
kept constant by adjusting the distance between the traps as the motor is packaging DNA. In fixed trap separation mode, the distance between the traps is kept fixed. A combination of both modes can also be used.

Materials

2.1 DNA substrate for stall-restart and in situ φ29 packaging

2.1.1 DNA
1. gp3-DNA (obtained from Drs. Shelley Grimes and Paul Jardine, described in Ref [11,43,44])
2. Lambda Phage DNA (500 µg/ml) (NEB, Inc.)

2.1.2 Nucleotides
1. Biotinylated (Biotin) forward 25.3 kbp primer: Biotin-5'
   CTGATGAGTTCGTGTCGTAAGCGGCACTGTAATC-3'
2. Digoxigenin (Dig) reverse 25.3 kbp primer: Dig-5'
   ATCCGATCTGGTACCAATGGATGGATG-3'
3. 12.2 kbp reverse primer: 5'-ACTCCGCCATGGCGTACGCACTGA-3'
4. 10 mM dNTP mix

2.1.3 Enzymes for DNA preparation
1. NcoI-HF (20 Units/µl) (NEB, Inc.)
2. T4 DNA Ligase (5 Units/µl) (Life Technologies)

2.1.4 Buffers
1. 10 mM TrisHCl pH 7.5
2. 10x NEB 2.1 Buffer (50mM NaCl, 10mM Tris-HCl, 10mM MgCl₂, 100µg/ml BSA, pH 7.9)
3. 5x T4 Ligase Buffer (250 mM Tris-HCl pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000)
2.1.5. DNA purification

1. Qiagen DNA purification kit (QIAquick PCR Purification Kit)
2. EMD Millipore Mixed Cellulose Ester Membranes: Diameter of 25 mm and pore size of 0.025 μm (used as dialysis pad for drop dialysis) (EMD Millipore #VSWP02500)

2.2 DNA substrate for T4 packing

The packaging substrate used for T4 packaging is the same substrate that is used for the \textit{in-situ} φ29 procedure (dual labeled 25,339 DNA). Here we list the materials required for preparing the 120 bp “primer” DNA.

2.2.1. DNA

1. Lambda phage DNA (NEB, Inc.)

2.2.2 Nucleotides

1. Forward 120 bp primer 5’-GACCATCACCCTGTATGAAGATTCACT-3’
2. Reverse 120 bp primer: 5’-ACCTCTTCCACCCATCAGTTCAAGAC-3’
3. 10 mM dNTP mix

2.2.4 Buffers

1. 10 mM TrisHCl pH 7.5

2.2.5 Materials for DNA purification by drop dialysis

1. EMD Millipore Mixed Cellulose Ester Membranes: Diameter of 25 mm and pore size of 0.025 μm (used as dialysis pad for drop dialysis) (EMD Millipore #VSWP02500)

2.3 Empty T4 complexes

2.3.1 Buffers

1. 10x T4 buffer: 800 mM NaCl, 250 mM Tris-HCl pH 7.5, 50 mM MgCl$_2$
2. Packaging buffer: 1x T4 buffer, 1 mM ATP, 0.1 mg/ml BSA

2.3.2 Proteins

1. T4 gp17 motor protein (~40 μM) (obtained from Dr. Venigalla Rao, described in Ref.)
2. T4 proheads or empty phage heads (~1.1e10/µl) (obtained from Dr. Venigalla Rao, described in Ref. [13])

3. Rabbit antisera prepared against T4 phages (obtained from Dr. Venigalla Rao, commercially prepared)

2.4 Empty or partially filled φ29 complexes

2.4.1 Solutions

1. 10x TMS buffer: 1 M NaCl, 500 mM Tris-HCl pH 7.5, 100 mM MgCl₂

2. Packaging buffer: 0.5x TMS, 0.5 mM ATP, 0.1 mg/ml BSA

2.4.2 Proteins

1. φ29 gp16 motor protein, diluted to ~0.1 µg/µl in 0.5x TMS (obtained from Drs. Shelley Grimes and Paul Jardine, described in Refs. [11,43,44])

2. φ29 proheads (with the associated pRNA), diluted to ~1 µg/µl in 0.5x TMS (obtained from Drs. Shelley Grimes and Paul Jardine, described in Refs. [11,43,44])

3. Antibodies against φ29 proheads (obtained from Shelley Grimes; prepared from Rabbit antisera by a commercial company and purified with a protein A column)

4. Superase-In RNase inhibitor (ThermoFisher Scientific, catalog #AM2696)

Materials and equipment needed for all three phage assays

1. 100 mM ATP in 100 mM Tris-HCl pH 7.5

2. 100 mM ATP-γ-S in 100 mM TrisHCl pH 7.5

3. TE (10 mM TrisHCl pH 8.0, 1 mM EDTA)

4. Purified deionized water (RNase, DNase, and Protease free)

5. 100 mg/ml Bovine Serum Albumin (BSA)

6. Streptavidin coated microspheres (~2.2 µm diameter, 0.5% w/v) (Spherotech, Inc.)

7. Protein-G coated microspheres (~2.2 µm, diameter 0.5% w/v) (Spherotech, Inc.)
8. Pipettors – 0.2-2 μl (P-2), 2-20 μl (P-20), 20-200 μl (P-200), 200-1000 μl (P-1000)
9. Dual optical tweezers instrument (described in Ref. [41])
10. Microcentrifuge (13,000 rpm/Max RCF ~13,000g)
11. 0.6 ml and 2 ml siliconized microcentrifuge tubes
12. 0.2 ml thin walled PCR tubes
13. 10 ml and 1 ml plastic syringes
14. Bucket of ice
15. Thermocycler
16. UV Spectrophotometer
17. Tube rotator – rotates at an angular velocity of ~1.25 rad/s
18. Long-range PCR kit (such as Extender system, 5 Prime, Inc.)

Methods

For all procedures that follow a P-2 pipettor is used when pipetting any volume below 2 μl, a P-20 pipettor is used when pipetting any volume > 2μl and ≤ 20μl, a P-200 pipettor is used when pipetting any volume > 20 μl and ≤ 200 μl, a P-1000 pipettor is used when pipetting any volume > 200 μl and ≤ 1000 μl.

3.1 φ29 DNA substrate for in situ packaging

3.1.1 Dual labeled 25.3 kbp DNA

1. Follow the procedure described in Section 3.6. Use Lambda phage DNA as the template DNA and use the 5’ end labeled biotinylated 25.3 kbp forward primer and 5’ end labeled digoxygenin 25.3 kbp reverse primer (Section 2.1.2).
2. After PCR, purify the DNA by drop dialysis against TE using the dialysis pad (Section 2.1.5)
3. Measure the DNA concentration with the UV spectrophotometer

3.2 φ29 DNA substrate for stall-restart packaging
3.2.1 Biotinylated 12.2 kbp DNA

1. Follow the procedure described in Section 3.6. Use Lambda phage DNA as the template DNA and use the 5’ end labeled biotinlyated 25.3 kbp forward primer and 12.2 kbp reverse primer (Section 2.1.2).

2. After PCR, purify DNA with the Qiagen mini-kit (This requires the centrifugation at ~13,000g)

3. Measure the DNA concentration with the UV spectrophotometer

3.2.2 Digestion of biotinylated 12.2 kbp DNA with NcoI

The final volume of this reaction is 50 μl.

1. Add 14 μl water to a 0.6 ml microcentrifuge tube

2. Add 5 μl 10x NEB 2.1 buffer

3. Add 30 μl of the purified biotinylated 12.2 kbp DNA (~169 ng/μl)

4. Add 1 μl NcoI-HF (20 Units/μl)

5. Mix by gently flicking the tube

6. Incubate at 37°C for 1 hour in a water bath

7. Purify the DNA with the Qiagen mini-kit (This requires the centrifugation at ~13,000g)

8. Measure the DNA concentration with the UV spectrophotometer

3.2.3 Digestion of gp3-DNA with NcoI

The final volume of this reaction is 20 μl.

1. Add 2 μl of water to a 0.6 ml microcentrifuge tube

2. Add 2 μl 10x NEB 2.1 buffer

3. Add 15 μl of the gp3 DNA (~450 ng/μl)

4. Add 1 μl NcoI-HF (20 Units/μl)
5. Mix by gently flicking the tube

6. Incubate at 37°C for 1 hour

7. Purify the DNA by drop dialysis against 10 mM Tris-HCl pH 7.5 using the dialysis pad for 30 min

8. Measure the DNA concentration with the UV spectrophotometer

3.2.4 Ligation of digested gp3-DNA and digested biotinylated 12.2 kbp DNA

The final reaction volume is 20 μl

1. Add 3.3 μl of water to a 0.6 ml microcentrifuge tube

2. Add 4 μl 5x T4 Ligase buffer

3. Add 1.2 μl digested gp3-DNA (~330 ng/μl)

4. Add 10.5 μl digested 12.2 kbp DNA (~95 ng/μl)

5. Add 1 μl T4 DNA Ligase (5 Units/μl)

6. Mix by gently flicking the tube

7. Incubate at 22°C for 1 hour in a water bath

8. Purify the DNA sample by drop dialysis against 20 mM TrisHCl pH 7.5 using the dialysis pad for 30 min

9. Store DNA in 4°C. (see Note 1)

3.6 PCR Procedure for generating DNA constructs for the phage tweezers packaging assays

First create the program shown in Table 3.1 on a thermocycler. Table 3.2 lists specific annealing and elongation temperatures and times as well as number of cycles for each respective phage DNA protocol.

Procedure for 100 μl PCR Reaction:

1. Start thermocycler program so that the block cools to 4°C and the lid heats to 105°C.
Be sure the program pauses and holds these temperatures until you are prepared to put the PCR samples on the block.

2. Have the following reagents thawed but cooled on ice:
   a. >5 μl of 10 mM dNTP mixture
   b. >1 μl of ~40,000 nM Forward & Reverse primer (see Note 1)
   c. >1 μl of the template DNA (50 ng/μl)
   d. >10 μl PCR Tuning Buffer W/Mg$^{2+}$ (see Note 2)
   e. >80 μl H$_2$O

3. Prepare two master mixes on ice according to the protocol in Table 3.3

4. Set four 0.4 μl PCR tubes on ice with their caps open.

5. Pipet the total volume of MM1 into the tube containing MM2. Then slowly pipet this mixture up and down ~10x for uniform mixture. (see Note 3)

6. Immediately pipet 25 μl of this into each of the PCR tubes on ice and close the caps tightly. (see Notes 4 & 5)

7. Immediately place these PCR samples in a well near the center of the thermocycler block. (see Note 5) Close the thermocycler lid, check that the lid snugly rests on the lids of the tubes, then unpause the thermocycler so that the program will resume.

8. Once the program is complete, 4 μl of each sample and 1 μl of λ-HindIII ladder is run on a 0.8% (w/w) 1x TAE agarose gel for 2.5 hours at 55V to check that the PCR was successful.

3.7 φ29 in situ tweezers packaging protocol

The assembly of stalled empty φ29 prohead-motor complexes requires purified proheads and purified gp16 monomers. After the motor assembles and binds to the prohead, the prohead-motor complex is stalled or stabilized by adding γ-S-ATP. Proheads and gp16 should be thawed, diluted to ~1 μg/μl and ~0.1 μg/μl in 0.5x TMS respectively, and kept on
ice before beginning the reaction. Use a siliconized 0.6 ml microcentrifuge tube. The biotinylated 25.3 kbp DNA needs to be prepared prior to beginning the procedure. (3.1). For this procedure the RCF of the micro-centrifuge, which is used to spin down the microspheres, is ~2000g.

3.7.1 Assembly of empty φ29 prohead-motor complexes

1. Pipet 4 µl of water to a 0.6 ml tube
2. Add 1 µl 10x TMS
3. Add 1 µl BSA (~2.5 mg/ml)
4. Mix the sample by gently flicking the tube
5. Add 2 µl proheads (~1 µg/µl)
6. Add 2 µl gp16 (~0.1 µg/µl) (see Note 6)
7. Mix the sample by gently flicking the tube
8. Incubate sample at room temperature for 2 min
9. Add 2 µl γ-S-ATP (3.5 mM)
10. Mix the sample by gently flicking the tube
11. Place tube on the tube rotator and let it rotate at room temperature for 60 min
12. Store the complexes at 4°C. They are typically usable for at most 24 hours.

3.7.2 φ29 antibody coated microspheres

The φ29 antibodies should be thawed and kept on ice before beginning this procedure.

1. Add 50 µl of protein G microspheres to a 0.6 ml tube
2. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min
3. Remove the supernatant
4. Wash the microspheres by resuspending them in 50 µl of 1x PBS
5. Repeat steps 2-4
6. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min
7. Remove the supernatant
8. Wash the microspheres by resuspending them in 5μl of 1x PBS
9. Add 1μl of φ29 antibodies (see Note 6) and mix the sample by gently flicking the tube
10. Place tube on the tube rotator and let it rotate at room temperature for 45 min
11. Add 45μl 1x PBS
12. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min
13. Remove the supernatant
14. Wash the microspheres by resuspending them in 50μl of 0.5x TMS
15. Repeat step 12-14
16. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min
17. Remove the supernatant
18. Wash the microspheres by resuspending them in 5μl of 0.5x TMS
19. Store the microspheres 4°C. Typically they are usable for ~1 week.

3.7.3 Binding of empty φ29 complexes to φ29 antibody coated microspheres
1. Add 5μl of 0.5x TMS to a 0.6 ml microcentrifuge tube
2. Add 2μl antibody microspheres
3. Mix the sample by gently flicking the tube
4. Add 4μl complexes (Section 3.7.1)
5. Mix the sample by gently flicking the tube
6. Place tube on tube rotator and let it rotate at room temperature for 60 min. Keep the sample on the rotator after this incubation to avoid the microspheres from settling to the bottom of the tube. Typically the samples are usable for at most ~6 hours.

3.7.4 Complex microsphere syringe
This step should be done immediately prior to beginning the optical tweezers experiment.

1. Add 500 μl of 0.5x TMS to a 2 ml tube
2. Add 4 μl of 20 mM γ-S-ATP
3. Add 4 μl complex microspheres
4. Mix the sample by gently flicking the tube
5. Suck solution into 1 ml syringe

3.7.5 Binding of biotinylated DNA to Streptavidin microspheres
1. Add 10 μl of Streptavidin microspheres (0.5 % w/v) to a 0.6 ml microcentrifuge tube
2. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min
3. Remove the supernatant
4. Wash the microspheres by resuspending them in 10 μl of 1x PBS
5. Add 30-50 ng of dual labeled 25.3 kbp DNA
6. Add 0.2 μl of 100 mg/ml BSA
7. Mix the sample by gently flicking the tube
8. Place tube on tube rotator and let it rotate at room temperature for 20 min
9. Store in 4°C. Typically these microspheres are usable for ~4 weeks.

3.7.6 DNA microsphere syringe
1. Add 500 μl solution of 0.5x TMS to a 2 ml microcentrifuge tube
2. Add 2 μl DNA microspheres
3. Mix the sample by gently flicking the tube
4. Suck solution into 1 ml syringe

3.7.6 φ29 packaging buffer
1. This buffer should be prepared fresh each day that experiments are performed and at room temperature. See materials (Section 2.4.1) above for the contents of this buffer.

3.8 φ29 stall-restart tweezers packaging protocol
The assembly of stalled partially filled φ29 prohead-motor complexes requires purified proheads, purified gp16 monomers, and biotinylated ligated ~27.2 kbp gp3-DNA. Packaging is initiated in bulk by adding ATP for ~20s and then stalling packaging by adding g-S-ATP. Proheads and gp16 should be thawed, diluted to ~1 μg/μl and ~0.1 μg/μl in 0.5x TMS respectively, and kept on ice before beginning the reaction. The ligated gp3-DNA needs to be prepared prior to beginning the procedure. (See Section 3.2). For this procedure the RCF of the micro-centrifuge, which is used to spin down the microspheres, is ~2000g. RNase inhibitor can optionally be added as a precaution to prevent possible degradation of the pRNA.

3.8.1 Assembly of partially filled φ29 prohead-motor complexes:

1. Add 1.5 μl water (volume before adding ATP (step 9) should be ~12 μl) to a 0.6 ml tube
2. Add 1 μl 10xTMS
3. Add 0.5 μl Superase-In RNase inhibitor
4. Add 5 μl ligated gp3-DNA (~20 ng/μl)
5. Add 2 μl proheads (~1 μg/μl)
6. Add 2 μl gp16 (~0.1 μg/μl)
7. Mix the sample by gently flicking the tube
8. Incubate sample at room temperature for 5 min
9. Add 2 μl ATP (3.5 mM) (ATP concentration = 0.5 mM) (see Note 6)
10. Mix the sample by gently flicking the tube
11. Incubate sample at room temperature for only 20s! (see Note 7)
12. Add 2 μl γ-S-ATP (7 mM)
13. Put complexes on ice for 5 min
14. Store at 4°C for future use. Complexes typically remain usable for at most ~24 hrs

3.8.2 φ29 antibody coated microspheres
1. Follow procedure 3.7.2

3.8.3 Binding of partially filled complexes to Streptavidin coated microspheres

1. Add 10 μl of Streptavidin microspheres (0.5% w/v) to a 0.6 ml tube
2. Pellet the microspheres by spinning them in a micro-centrifuge for ~1-2 min
3. Remove the supernatant
4. Wash the microspheres by resuspending them in 10 μl of 0.5x TMS
5. Repeat Steps 2-4
6. Add 4 μl of partially filled complexes sample (~7 ng/μl, see Note 8)
7. Mix the sample by gently flicking the tube
8. Place tube on tube rotator and let it rotate at room temperature for 20 min
9. Leave tube on rotator for future use. The complexes are typically usable for at most ~6 hours.

3.8.3 Complex-DNA microsphere syringe

1. Add 500 μl solution of 0.5x TMS to 2 ml tube
2. Add 4 μl of 20 mM γ-S-ATP
3. Add 4 μl complex-DNA microspheres
4. Mix the sample by gently flicking the tube
5. Suck solution into 1 ml syringe

3.8.4 Preparation of antibody microsphere solution

1. Add 500 μl solution of 0.5x TMS to a 2 ml tube
2. Add 2 μl antibody microspheres
3. Mix the sample by gently flicking the tube
4. Suck solution into 1 ml syringe

3.8.5 φ29 packaging buffer: This buffer should be prepared fresh each day that experiments are performed and at room temperature. See materials (Section 2.4.1) above for the
The assembly of T4 head-motor complexes requires purified heads, purified gp17 monomers, and purified 120 bp DNA (see Note 8). The complex is stabilized by adding γ-S-ATP. Heads and gp17 should be thawed and kept on ice before beginning the reaction. Both the dual labeled 25.3 kbp DNA (Section 3.1) and the 120 bp DNA (Section 3.4) must be made prior to beginning this protocol. For this procedure the RCF of the micro-centrifuge, which is used to spin down the microspheres, is ~2000g.

3.9.1 T4 antibody microspheres

The T4 antisera should be thawed and kept on ice before beginning this procedure.

1. Pipet 50 μl of protein G microspheres to a 0.6 ml microcentrifuge tube
2. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min
3. Remove the supernatant
4. Wash the microspheres by resuspending them in 50 μl of 1x PBS
5. Repeat step 2-4
6. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min
7. Remove the supernatant
8. Wash the microspheres by resuspending them in 5μl of 1x PBS
10. Add 1 μl of T4 antisera and mix the sample by gently flicking the tube
9. Place tube on tube rotator and let it rotate at room temperature for 45 min
10. Add 45 μl 1x PBS
11. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min
12. Remove the supernatant
13. Wash the microspheres by resuspending them in 50 μl of 1x T4 buffer
14. Repeat step 12-14
15. Pellet the microspheres by spinning them in a microcentrifuge for ~1-2 min

16. Remove the supernatant

17. Wash the microspheres by resuspending them in 5 μl of 1x T4 buffer

18. The microspheres should be stored at 4°C and are typically usable for at most 1 week.

3.9.2 T4 complexes

The final volume of the reaction is ~12 μl.

1. Add 2 μl 3x T4 buffer to a 0.6 ml tube

2. Add 1 μl 20 mM γ-S-ATP

3. Add 4 μl 120 bp DNA (~200 ng/μl)

4. Add 2 μl gp17 (~40 μM)

5. Add 3 μl T4 heads (~1.1e10/μl)

6. Mix the sample by gently flicking the tube

7. Place tube on tube rotator and let it rotate at room temperature for 45 min

3.9.3 T4 complex microspheres

1. Add 1.5 μl of T4 coated antibody microspheres to T4 complex solution (Section 3.9.2)

2. Mix the sample by gently flicking the tube

3. Place tube on tube rotator and let it rotate at room temperature for 45 min

4. Leave sample on rotator for future use. Samples are typically usable for at most ~6 hours.

3.9.4 T4 complex microsphere syringe solution

1. Pipet 500 μl solution of 1x T4 buffer to a 2 ml microcentrifuge tube

2. Add 4 μl of 20 mM γ-S-ATP

3. Add 4 μl complex microspheres

4. Mix the sample by gently flicking the tube
5. Suck solution into 1 ml syringe

3.9.5 DNA microspheres: Since T4 uses the same packaging construct as φ29, follow the procedure described in Section 3.7.5.

3.9.6 DNA microsphere solution
1. Pipet 500 μl solution of 1x T4 buffer to a 2 ml microcentrifuge tube
2. Add 2 μl DNA microspheres
3. Mix the sample by gently flicking the tube
4. Suck solution into 1 ml syringe

3.9.7 T4 packaging buffer: This buffer should be prepared fresh each day that experiments are performed and at room temperature. See materials (Section 2.3.1) above for the contents of this buffer.

3.11 Microsphere capture with optical tweezers and initiation or re-starting of packaging complexes

The microsphere solutions and packaging buffer are injected into the flow chamber (Fig. 1.1). Add enough microsphere solution into the flow chamber until the microspheres are visibly flowing out of the capillary tube. The packaging solution is injected last. Using a tube-crimping "valve" on the polyethylene tubing that inserts into the main channel of the flow chamber, adjust the flowrate in the central channel to ~ 1 μm/ to sweep away excess microspheres.

3.11.1 Microsphere capture for in situ initiation of empty prohead complexes

This procedure is for capturing empty T4 and φ29 prohead-motor complexes. The top chamber contains DNA microspheres and the bottom chamber contains complex microspheres (Fig. 3.2A).
1. Capture the DNA microsphere with the fixed trap
2. Capture complex microsphere with the movable trap
3.11.2 Microsphere capture for re-starting packaging of stalled complexes

This procedure is for capturing partially filled λ and φ29 prohead-motor complexes. The top chamber contains complex-DNA microspheres and the bottom chamber contains antibody microspheres (Fig. 3.2B).

1. Capture the antibody microsphere with the fixed trap
2. Capture complex-DNA microsphere with movable trap

After microsphere capture, a fishing procedure is implemented (Fig. 3.2 A&B). We automate the procedure by building it into our data acquisition and instrument control program. Thus after the microspheres are captured we let our program search for a tether hook-up.

1. Record background force: Record the force signal of the fixed trap without tethered microspheres for 1s. The average value is the background force or value of the force signal with no applied force.
2. Tether hook-up: Decrease the distance to ~1 μm and hold for 1 s (see Note 9) to initiate packaging or attachment of DNA-complex tether.
3. Check for packaging: Increase the distance to the beginning of the expected range of where packaging should start. We often measure non-specific tethering of DNAs with apparent length much shorter than expected tether lengths. In this case, when fishing for the desired length DNA construct, we find that the traps can be moved apart to rupture such tethers. Typically, we set the range minimum to equal 1 μm less than the packaging construct tether length.
4. Apply measurement mode: If the force rises to ~5 pN and the distance between the microspheres is within the expected range, the program enters into either “force-clamp”, “fixed trap separation” or a combination of both modes (depending on the choice of the experimenter). The force is monitored by subtracting the background
force from the current force reading.

3.12 Modes of detection for single molecule packaging experiments: force clamp and fixed trap separation

3.12.1 Force clamp mode:

The data acquisition program maintains a constant force by adjusting the relative distance between the microspheres by a fixed step size in real time. The distance is increased/decreased if the force is less/more than the force clamp set point. The loop or sample rate is typically set to 1 kHz and the roll-off frequency of our analog low pass anti-aliasing filter is set to 330 Hz. The step size of the clamp is determined by requiring that the maximum step rate to be at least ~2x greater than the maximum DNA translocation rate.

3.12.2 Fixed trap separation mode:

The distance between the two optical traps is held constant causing the force to increase with increasing length of DNA packaged. Since our microspheres, which have a diameter of 2.2 μm, exhibit a corner frequency (effective measurement bandwidth due to hydrodynamic drag).

4. Notes

1. Gp3-DNA that has been dialyzed against 10 mM TrisHCl pH 7.5 can typically be used for only 1 week because the gp3 protein can degrade.

2. One may note that figure 3.1 does not show a digoxigenin label. This is because the digoxigenin is not important to the packaging experiment. However, we typically do include both a digoxigenin and biotin label on opposite ends of the dsDNA so that we can confirm the DNA has been properly generated by tethering the DNA between streptavidin and anti-digoxigenin microspheres and stretching it in the optical tweezers to verify that the tethers are the expected length.

3. We typically request our primers to be sent dry so that we can suspend them in the buffer of
our choosing and with the concentration that is best for our work.

4. This is the buffer that comes with the 5 Prime PCR Extender System.

5. Since the polymerase is stored in glycerol, bubbles can easily form when combining the master mixes. Bubbles can disrupt uniform mixing and heating, which can disturb the PCR reaction. To maximize the PCR yield, be careful not to introduce bubbles when mixing the master mixes.

6. Avoid introducing bubbles while pipetting each PCR sample in this step.

7. Samples of 20-25 μl also work and, in fact, this is the volume we typically use.

8. While this may not matter for robust PCR or all thermocyclers we place the samples near the center of the thermocycler block where we expect the temperature to be most accurately controlled. The temperature of the wells at the edge of the block in some thermocyclers may not be consistent with the temperature of the internal wells because of their exposure to the air and/or insulating material.

9. If the packaging efficiency is low we find that it sometimes helps to increasing the amount of added gp16 by up to several-fold.

10. We have used both purified (IgG) and unpurified antibodies from antisera during this step and both work equally well. Notably, the process of coating protein G microspheres with unpurified antisera and washing is effectively a means of purification, which presumably leaves only antibodies attached to the microspheres.

11. To package a lower fraction of the genome in bulk, add a lower ATP concentration. For example, to package ~10% of the genome, we typically add 0.35 mM ATP such that the final ATP concentration is ~0.05 mM. However in this condition the efficiency may be reduced several-fold.

12. To package a larger fraction of the genome in bulk, increase the ATP incubation time. For example, to package ~50% of the genome, we usually wait ~1 minute.
13. If the efficiency in the tweezers is low, we sometimes add \( \sim 7 \mu l \) of complexes to 10 \( \mu l \) of streptavidin microspheres. We find that this increases the efficiency slightly.

14. The 120 bp DNA is not required but we find that adding it significantly increases the efficiency by as much as an order of magnitude.

15. Often after centrifugation there exists a layer of microspheres at the liquid surface, which, when extracting the supernatant, swirls down into the supernatant. For consistent preparation of washed microspheres, if such a layer of microspheres is observed to swirl down into the supernatant, one can stop pipetting at this instance and centrifuge the tube once again. Typically after doing so, the microspheres at the liquid surface and those that had been suspended in the supernatant will have pelleted to the bottom of the microcentrifuge tube. After this step the remaining supernatant can then be pipetted out. This note can be applied to all instances of microsphere washing in this chapter.

16. The use of BSA reduces aggregation of streptavidin microspheres.

17. We have found that these microspheres have a tendency to adhere to the walls of the microcentrifuge tube and that both aggressive washing (by up and down pipetting action) and the coating with \( \lambda \) antisera (step 8) tend to increase the adherence of these microspheres to the tube walls. Notably, this adsorption tends to be considerably greater after the addition of the \( \lambda \) antisera. The adherence of microspheres to the tube walls substantially reduces the final concentration of prohead antibody microspheres. To maximize the final concentration of prohead antibody microspheres, we minimize the amount of pipetting necessary to achieve an apparent uniform mixture of microspheres during the washing steps and also avoid aggressive pipetting. Occasionally, if we are suffering from excessive microsphere adsorption, despite employing these measures, we will eliminate one of the wash steps requested in step 11 and/or step 12 in this section of the protocol (3.10.2). Such procedures also help to produce more consistently prepared
antibody coated microspheres.

18. The DNA is not purified after PCR because we find that this does not appear to affect the efficiency of DNA packaging. However, if one needs to know the concentration of the DNA, it can be purified by dialyzing it against 10 mM TrisHCl pH 7.5 and checking the concentration with the UV spectrophotometer as in step 3 of Method 3.1.

19. The terminase extract contains glycerol. As a consequence of adding this in this prep, bubbles can easily form by accident when mixing and/or pipetting. Avoid generating bubbles because bubbles will inhibit uniform mixing and subsequently inhibit formation of DNA-terminase-prohead complexes.

20. This step could probably be shorter, but it doesn’t hurt to have a longer than necessary incubation time here. This incubation is simply done to provide all the reagents in the tube time to diffuse throughout the mixture if they have yet to be uniformly distributed from previous mixing steps.

21. In this protocol the ratio of ATP to γ-S-ATP is 1:1. However, the amount of DNA that has prepackaged before restarting packaging within the tweezers can potentially be reduced by adjusting this ratio (particularly by increasing the amount of γ-S-ATP relative to the amount of ATP). Moreover, the amount of prepackaged DNA can likely be reduced by decreasing the incubation time during the steps that follow this step (i.e. step 9, 11, and Section 3.10.4 – step 4).

22. The volume of complexes we use here typically ranges from ~0.3-1 μl depending on the activity of the reagents, which is identified based on the observed efficiency of obtaining complexes within the tweezers that package DNA (Section 3.11).

23. Typically we find that the efficiency of measuring packaging events in the tweezers decreases with time. Complexes can often still be acquired after 6 hours, and in some cases after 24 hours, however the activity is typically substantially reduced by this time.
24. The volume of microspheres added here depends on the concentration of the microspheres and how many microspheres the tweezers operator would prefer flowing out of the capillary tubes within the flow cell during experiments. Too few microspheres make the capture of microspheres in the flow cell inefficient while a large excess of microspheres exiting the capillary tubes make it difficult to capture a single microsphere without another colliding into it before moving to the opposing capillary tube. Given the microsphere concentrations achieved after our antibody microsphere preps, we rarely add more than 5 μl to this syringe solution.

25. No activity in the tweezers will be observed if γS-ATP is absent in this syringe solution.

26. We find that increasing the time to 2-3 seconds increases the efficiency by ~50%.

27. The maximum force achieved is typically 40 pN because above 40 pN the linkage between the antibody and prohead breaks rapidly (usually within tens of seconds), although higher forces can be applied for a shorter time.

Chapter 3, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in Methods in Molecular Biology. N. Keller, D. E. Smith. (2016). Single-molecule measurements of motor-driven viral DNA packaging in bacteriophages phi29, lambda, and T4 with optical tweezers.
Table 3.1 Overview of Steps.

<table>
<thead>
<tr>
<th>Step</th>
<th>Task</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prepare DNA substrates</td>
</tr>
<tr>
<td>2</td>
<td>Assemble complexes</td>
</tr>
<tr>
<td>3</td>
<td>Attach complexes to microspheres</td>
</tr>
<tr>
<td>4</td>
<td>Inject microspheres into flow chamber</td>
</tr>
<tr>
<td>5</td>
<td>Capture microspheres with optical traps</td>
</tr>
<tr>
<td>6</td>
<td>Tether complexes</td>
</tr>
<tr>
<td>7</td>
<td>Initiate and measure packaging</td>
</tr>
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</table>
Table 3.2 PCR Parameters.

<table>
<thead>
<tr>
<th>Stage #</th>
<th>Step</th>
<th>Temp.</th>
<th>Time</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Heated lid:</td>
<td>105ºC</td>
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<td></td>
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<tr>
<td>Preheated lid:</td>
<td>on</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample cooling (4ºC):</td>
<td>on</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pause:</td>
<td>on</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Stage 1 10 cycles (skip for 120 bp DNA)</th>
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<th>93 ºC</th>
<th>15 sec</th>
<th>Template denaturation</th>
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<tr>
<td></td>
<td>3</td>
<td>T_Anneal</td>
<td>30 sec</td>
<td>Primer annealing</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>T_Elong</td>
<td>t_Elong</td>
<td>Primer extension/elongation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage 2 N_cycles cycles</th>
<th>5</th>
<th>93 ºC</th>
<th>15 sec</th>
<th>Template denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>T_Anneal</td>
<td>30 sec</td>
<td>Primer annealing</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>T_Elong</td>
<td>t_Elong + 20sec/cycle</td>
<td>Primer extension/elongation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Final extension:</th>
<th>off</th>
</tr>
</thead>
</table>

| Stage 2          | 8  | 4ºC  | -- | Final Hold |

Hot start: off
Table 3.3 PCR Mixtures.

<table>
<thead>
<tr>
<th>DNA construct</th>
<th>λ Biotinylated 13.7 kbp</th>
<th>T4 120 bp</th>
<th>Dual labeled 25.3 kbp</th>
<th>Biotinylated 12.2 kbp</th>
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</thead>
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<tr>
<td>T\textsubscript{Anneal (°C)}</td>
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<td>60</td>
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<td>68</td>
</tr>
<tr>
<td>t\textsubscript{Elong (min)}</td>
<td>11</td>
<td>30</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>N\textsubscript{cycles (cycles)}</td>
<td>18</td>
<td>30</td>
<td>18</td>
<td>18</td>
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### Table 3.4 Preparation of PCR Mixtures.

<table>
<thead>
<tr>
<th></th>
<th>Template DNA</th>
<th>Final Conc.</th>
<th>Procedural Notes</th>
</tr>
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<tbody>
<tr>
<td>Master Mix 1</td>
<td>40 μl</td>
<td></td>
<td>Combine in a 0.6 ml microcentrifuge tube</td>
</tr>
<tr>
<td>(MM1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 H₂O</td>
<td>37 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Forward Primer</td>
<td>1 μl</td>
<td>400 nM</td>
<td>Use ~40,000 nM</td>
</tr>
<tr>
<td>3 Reverse Primer</td>
<td>1 μl</td>
<td>400 nM</td>
<td>Use ~40,000 nM</td>
</tr>
<tr>
<td>4 Template DNA</td>
<td>1 μl of 50 ng/μl</td>
<td>~0.5 ng/μl</td>
<td>After combining all ingredients, slowly pipet this mixture up and down approximately 10x to ensure uniform mixing, then place the mixture on ice.</td>
</tr>
</tbody>
</table>

|                |              |             |                                                                                   |
| Master Mix 2   | 60 μl        |             | Combine in another 0.6 μl microcentrifuge tube                                     |
| (MM2)          |              |             |                                                                                   |
| 1 H₂O          | 44.2 μl      |             |                                                                                   |
| 2 10x Tuning Buffer W/Mg₂⁺ | 10 μl | 1x; 2.5 mM Mg²⁺ |                                                                                   |
| 3 10mM dNTP mix | 5 μl     | 500 μM      |                                                                                   |
| 4 5 Prime PCR Extender Polymerase Mix | 0.8 μl | 0.04 Units/μl | Remove the 5 Prime PCR Extender Polymerase Mix from the freezer, quickly pipet 0.8 μl of polymerase, quickly put the stock polymerase back in the freezer, then slowly pipet the polymerase into this master mix while slowly swirling the pipet tip throughout the master mix solution. *(see Note 1)* Lightly flick the mixture for ~10-20 seconds for additional mixing then place the mixture on ice. |
Figure 3.1 Flowcell. Schematic illustration of the flow chamber used in the experiments. The top and bottom channels are used to introduce microspheres and are connected to the center channel by microcapillary tubes. The optical tweezers trap microspheres in the center channel where the measurements are performed.
Figure 3.2 Capturing Microspheres. Schematic illustration of how the measurements of single DNA molecule packaging are performed. (A) The in situ initiation method. (B) The stall-restart method. See 3.11.3 Fishing procedure for initiation of packaging or attachment of a DNA-complex tether.
References


Chapter 4

Nonequilibrium Dynamics and Ultraslow Relaxation of Confined DNA during Viral Packaging
**Abstract**

Many viruses employ molecular motors that generate large forces to package DNA to near crystalline densities inside preformed viral proheads. Besides being a key step in viral assembly, this process is of interest as a model for understanding the physics of charged polymers under tight 3D confinement. A large number of theoretical studies have modeled DNA packaging, and the nature of the molecular dynamics and the forces resisting the tight confinement is a subject of wide debate. Here, we directly measure the packaging of single DNA molecules in bacteriophage phi29 with optical tweezers. Using a new technique in which we stall the motor and restart it after increasing waiting periods we show that the DNA undergoes nonequilibrium conformational dynamics during packaging. We show that the relaxation time of the confined DNA is $>10$ minutes, which is longer than the time to package the viral genome and $60,000 \times$ longer than that of the unconfined DNA in solution. Thus, the confined DNA molecule becomes kinetically constrained on the timescale of packaging, exhibiting glassy dynamics which slows the motor, causes significant heterogeneity in packaging rates of individual viruses, and explains the frequent pausing observed in DNA translocation by the motor. These results support several recent hypotheses proposed based on polymer dynamics simulations and show that packaging cannot be fully understood by quasi-static thermodynamic models.

**Introduction**

DNA packaging is both a critical step in viral assembly and a unique model for understanding the physics of polymers under strong confinement. Prior to packaging, the DNA (~6-60 µm long) forms a loose random coil of diameter ~1-3 µm. After translocation into the viral prohead (~50-100 nm in diameter) a ~10,000-fold volume compaction is achieved. Packaging is driven by a powerful molecular motor that must work against the large
forces resisting confinement arising from DNA bending, repulsion between DNA segments, and entropy loss (1-8).

DNA packaging in bacteriophages phi29, lambda, and T4 has been directly measured via single molecule manipulation with optical tweezers and the packaging motors have been shown to generate forces of >60 pN, among the highest known for biomotors, while translocating DNA at rates ranging from ~100 basepairs (bp) (for phage phi29, which packages a 19.3 kbp genome into a 42×54 nm prohead shell) up to as high as ~2000 bp/s (for phage T4, which packages a 171 kbp genome into a 120×86 nm prohead) (9-15). The force resisting packaging rises steeply with prohead filling, and has been proposed to play an important role in driving viral DNA ejection (16).

Recently, a variety of theoretical models for viral DNA packaging have been proposed (3-5, 17-21). The simplest treat DNA as an elastic rod with repulsive self-interactions and assume that packaging is a quasi-static thermodynamic process, i.e., that the DNA is able to continuously relax to a free-energy minimum state (3-5, 19-21). The DNA arrangement is generally assumed to be an inverse spool with local hexagonal close packing between DNA segments, as suggested by electron microscopy and X-ray scattering studies (22, 23). Such models yield exact analytical predictions that reproduce many of the experimental trends, including the sharp rise in resistance during the latter stages of packaging (3-5, 20).

Dynamic simulations, on the other hand, predict differing results. Depending on model and simulation protocol, some predict rapid equilibration into ordered spool or folded toroid conformations, while others predict nonequilibrium dynamics and disordered conformations (3, 6, 24-31). The packaged DNA conformation also depends on ionic conditions, capsid size and shape, and shape of the internal core structure found in some phages (6, 30). Notably, some electron microscopy studies have also been interpreted as suggesting ordered spooled conformations (22), while others interpreted as suggesting partly
disordered conformations (29). While some simulations predict nonequilibrium dynamics, several potential caveats are that: (i) the DNA has been represented by coarse-grained polymer models with various approximations for physical interactions (6), (ii) the packaging rate used in the simulations is \(>10^5\) higher than the measured packaging rate due to computational constraints (3, 26-28), and (iii) it has been pointed out by some authors that simulation timescale cannot be directly related to experimental timescale because of the use of coarse-grained models for DNA (25, 28). As noted in early modeling studies, the calculations based on quasi-static models may represent a lower-bound on the required packaging forces due to dissipative dynamic losses (4). Whether non-equilibrium dynamics play a significant role in real systems has thus remained an important open question.

**Results**

We measured the packaging of single DNA molecules into single phage phi29 proheads using a modified version of the optical tweezers technique described previously (10, 13) (see also Materials and Methods). Briefly, complexes consisting of the prohead with bound gp16 ATPase motor protein are attached to one microsphere and DNA molecules are attached to a second microsphere. When the DNA is brought into contact with the motor in the presence of ATP, packaging initiates and the DNA is translocated into the prohead (Fig. 1A). Packaging measurements were made with an applied 5 pN force, which is small compared with the maximum force the motor can exert (>60 pN). The phi29 motor slows as packaging proceeds due to building forces resisting DNA confinement.

To test for nonequilibrium dynamics, we developed a new method (Fig. 1A) in which packaging was initiated and followed to \(~75\%\) prohead filling, where the motor has slowed significantly (9, 10, 13). We then stall the motor with non-hydrolyzable ATP and, after an imposed waiting time, restart packaging by re-introducing ATP. If the DNA were in a
nonequilibrium state one would expect it to relax during the stall towards a lower energy state, thus presenting less resistance and, enabling the motor to accelerate upon restarting.

Indeed, measurements revealed clear increases in packaging rate upon restarting, providing direct evidence for nonequilibrium dynamics. A typical example is shown in Fig. 1B&C where the rate increased 47% after a 10.4 minute long stall. The average changes in packaging rate and motor velocity (rate not including pauses and slips, $\langle \Delta \nu \rangle$) clearly increase with increasing stall time (Fig. 1D). That both quantities exhibit significant changes illustrates that the acceleration is due mostly to a change in the inherent motor velocity, not just due to decreases in pausing and slipping. As a control, the same analysis procedure applied to events without induced stalls revealed no change in motor velocity, on average. This test shows that the observed accelerations following stalls are not simply due to normal fluctuations in motor velocity. Measurements after stalling at $<50\%$ filling also show no acceleration ($\langle \Delta \nu \rangle = -4\%$; n= 85), indicating that nonequilibrium effects only have a measurable effect when the DNA is densely packed.

The observed acceleration after stalling implies a reduction in load on the motor due to relaxation of the DNA to lower energy conformations presenting lower resistance. We note that our previous studies employed the measured velocity vs. applied force relationship to infer an effective "internal force" resisting packaging (9, 10, 13). However, recent studies indicate that while both prohead filling and applied force slow the motor, they have a different influence on the detailed motor stepping kinetics and thus cannot be directly equated (32). Here, we refer to "resistance" or "load" on the motor as any interaction between the packaged DNA and motor which slows packaging.

The increasing trend of $\langle \Delta \nu \rangle$ with increasing stall time indicates that 10 minutes is a lower bound on the DNA relaxation time. Longer stalls were attempted, but we found measurements of the motor restart could rarely be obtained. The finding that nonequilibrium
conformations persist for longer than it takes to package the entire genome (~6.6 min, on average) implies that the DNA can become kinetically trapped in an inherently nonequilibrium or "glassy" state. Thus, packaging is a dissipative process in which the motor must do more work than if the DNA was able to continuously relax to a minimum energy state.

Another expected consequence of nonequilibrium dynamics is heterogeneity in the dynamics of individual complexes. To investigate this we analyzed an ensemble of packaging events without imposed stalls (Fig. 2A). Inherent variability in packaging rate is expected even in the absence of a load due to stochastic motor kinetics; at low filling this is characterized by a constant index of dispersion (variance in motor velocity divided by mean velocity), as shown by detailed studies of the motor function (33). Consistent with this, we find a constant index of dispersion up to 40% filling, but observe a clear increase beyond this point to a final value ~10-fold higher than that at low filling (Fig. 2B). This increase in dispersion indicates that the heterogeneity is due to variability in resistance to DNA confinement between different individual complexes. This finding implies the DNA does not follow a single conformational pathway during packaging.

Two additional findings further support this conclusion. First, a small fraction of packaging events do not show the usual trend of velocity decreasing with filling and the motors translocate far past the genome length (Fig. 2A). We interpret these as cases where the prohead is perforated and the DNA leaks out, so there is no resistance to packaging. A similar effect was observed with phage lambda proheads lacking a stabilizing protein (11). Notably, for these events the dispersion did not increase with length of DNA translocated. Second, the dispersion is also reduced following imposed stalls (Fig. 2B), consistent with the reaction proceeding closer to equilibrium following relaxation of the packaged DNA.

Our results imply that the DNA can reorganize during packaging, despite being confined at very high density. A question of interest is what physical factors influence the
DNA mobility. It has been proposed that Coulomb sliding friction between DNA segments may play an important role at high filling densities (18). The measurements described above were in conditions with Na\(^+\) and Mg\(^{2+}\) ions screening the DNA, where the interaction between closely packed segments is purely repulsive (4, 20). We also analyzed packaging measurements with 5 mM of added Spermidine\(^{+3}\), which induces a partly attractive interaction between DNA segments (34). In this case, we find a much larger index of dispersion (Fig. 2D), indicating that this condition favors the trapping of highly nonequilibrium conformations. Thus we have shown that the magnitude of the nonequilibrium effect can be reduced (by allowing relaxation), or increased (by changing the nature of the DNA-DNA interaction). Notably, studies have also shown that spermidine can inhibit DNA ejection from phage heads (16, 35), consistent with the notion that the DNA is locked in a condensed conformation.

A previously observed, but unexplained feature in phi29, lambda, and T4 packaging is the occurrence of ~1-10 second pauses in DNA translocation (9, 11, 12). Pausing increases sharply at high filling (Fig. 3A), suggesting it is connected to build-up of resistance. However, since the pauses are transient it is clear that the average resistance is not sufficiently high to continuously stall the motor. It has been proposed, based on simulations, that pausing could be due to large fluctuations in resistance due to formation of local nonequilibrium DNA conformations that transiently block packaging (26, 36). Consistent with this prediction, we observe that the average pause duration increases significantly above ~70% filling (Fig. 3A), suggesting conformations that temporarily prevent packaging relax on a timescale that increases with packaged DNA density. Notably, however, pause durations are much shorter than the >10 minute relaxation time revealed by our stall experiments. Thus, in our experiments there is evidence for two relaxation timescales: one associated with pauses lasting seconds, presumably corresponding to relaxation of locally jammed conformations, and a
second associated with relaxation of the average resistance on a timescale >10 minutes, presumably corresponding to global relaxation.

We also observe a striking decrease in both frequency and duration of pauses following imposed stalls (Fig. 3), showing that pauses are associated with nonequilibrium conformations and suggesting that the two relaxation processes are experimentally coupled. Long pauses (> 5 s) are virtually eliminated following long stalls, further supporting the conclusion that DNA relaxation facilitates faster ongoing packaging via a closer-to-equilibrium conformational pathway. In addition, we observed virtually no pausing in the events where packaging did not slow with filling. In contrast, addition of spermidine causes a significant increase in the amount of time spent paused, consistent with increased trapping of the DNA in nonequilibrium conformations.

**Discussion**

Our findings show that packaging cannot be fully understood by quasi-static thermodynamic models and support several hypotheses proposed based on dynamic simulations. Forrey and Muthukumar predicted a significant influence of nonequilibrium DNA polymer dynamics on viral packaging (26). Specifically, consistent with our findings, they predicted large heterogeneity in dynamics at >50% filling, large fluctuations in resistance forces, and pauses in DNA translocation. They attributed pauses to steric blockages due to local crowding of DNA segments and pause durations to relaxation dynamics of the confined DNA, predicted to adopt a nonequilibrium glassy structure at high filling.

Ali et al. also predicted disordered conformations and pauses with durations that increase with filling due to fluctuations in resistance (27, 36). Additionally, they predicted that the forces driving viral DNA ejection, occurring at a later time after packaging has finished when a virus infects the next cell, would be lower than the forces resisting packaging due to dissipative dynamics. The latter prediction is consistent with our finding that when packaging
is stalled the DNA can relax to a conformation presenting less resistance. Petrov and Harvey also predicted varying conformations and resistance at high filling and the need for extensive equilibration to produce partially ordered structures (6, 30). In addition they predicted, from analysis of structural correlation functions, that the DNA would adopt a glassy state at high filling (31).

Available experimental information on DNA structure in phi29 has come from cryo-electron microscopy studies in which 3D reconstructions are obtained by averaging a large ensemble of images of individual frozen particles (37, 38). While early studies of the DNA conformation in phage T7 were interpreted as indicating a highly ordered spool conformation of DNA based on the observation of distinct layers of average DNA density, later studies of other phages and modeling studies called the generality of this interpretation into question (26, 29). Comolli et al. studied phi29 by cryo-electron microscopy with 3D reconstruction from ensembles of images of frozen particles (29). With a fully packaged genome the arrangement of a central portion of DNA in the core was unresolved but five concentric layers of average DNA density adjacent to the prohead wall were observed, suggesting local close packing of segments in this region. However, reconstructions of partially packaged (32-78%) proheads showed uniform average DNA density throughout, consistent with heterogeneous DNA conformations.

Beyond having relevance to viral packaging, the behavior of polymers in confined geometries has long been a topic of fundamental interest in polymer physics (39-41) and, more recently, has attracted a great deal of attention due to emerging applications of nanofabricated devices for DNA manipulation in biotechnology (42). Confinement of single DNA molecules in nanofabricated slits and channels has been extensively studied and shown to increase the molecular relaxation time due to reductions in conformational entropy and hydrodynamic interactions with the confining walls (42-44). Entropic penalties for confining flexible or
wormlike polymers in slits, tubes, and spheres have recently been computed theoretically (45). Experimentally, in the most extreme cases reported, confinement of lambda DNA between two surfaces separated by 32 nm was shown to increase the molecular relaxation time ~30-fold (44), while confinement in a 140×130 nm channel caused a ~20-fold increase (42, 43). The effect of tight 3D confinement has not previously been experimentally studied but our results show that it causes more dramatic effects. Specifically, the >10 minute relaxation time revealed by our experiments is >60,000× that for unconfined DNA of the same length (46).

The DNA concentration within phi29, like many other double-stranded DNA bacteriophages, is very high (~0.5 g/ml) and one would expect excluded volume and chain entanglements to strongly restrict molecular motion (39, 47, 48). The effect of entanglements on the dynamics of polymers in melts and concentrated solutions has been successfully predicted by reptation models, in which polymer motion is restricted to a tube-shaped region parallel to the chain contour (39, 49). Viral DNA packaging differs in several ways from the typical situations in which the reptation model is applied. Specifically, one end of the DNA is constrained by the motor, it is a highly charged polymer tightly confined in three dimensions, and at the highest densities it may form a variety of locally-ordered liquid crystal-like phases (18, 50), all of which may further slow relaxation. Nevertheless, the notion that a reptation-like process may play a role in viral DNA packaging, or in the converse process of DNA ejection, has been discussed previously in a number of theoretical papers (18, 51-53). It has been proposed, for example, that the relaxation time predicted by reptation theory may set a lower bound on the relaxation time of the packaged DNA (54). The tube disengagement time in reptation theory is predicted to scale with polymer length (L) and concentration (c) as $\tau_D \sim L^3 c^{1.5}$ (39). Studies in our lab indicate that $\tau_D \approx 40$ s for 115 kbp DNA at 1 mg/ml (49). For the length and concentration of DNA confined in the phi29 prohead at 75% filling the
theoretical scaling law would thus predict $\tau_D=10.6$ minutes, a value consistent with the lower-bound implied by our stall-restart measurements.

In summary, we report evidence for nonequilibrium dynamics of DNA during viral packaging that slows the motor, causes heterogeneity in packaging rates of individual viruses, and explains the frequent pausing observed in motor translocation. Because many viruses package DNA to similarly high density as phi29 it is likely that our findings are broadly applicable. Studies of several phages, including phi29, show that DNA is packaged in vivo (in the infected host cell) at similarly high rates as we measure in vitro (55-57). Therefore, the nonequilibrium effects we report are likely relevant in vivo. Although fast packaging may result in higher mechanical work due to nonequilibrium forces, the motor is nevertheless strong enough to complete the process. Faster packaging also does not necessarily imply a higher energy requirement, because studies and models suggest that the motor utilizes the same amount of ATP per total length of DNA packaged regardless of load and packaging rate (33, 58). Our studies also provide unique experimental data to test theoretical models of viral packaging; and, more generally, theories for polymer dynamics under strong 3D confinement.

Materials and Methods

Fiberless proheads were produced by infection of B. subtilis 12A (sup-) with the phi29 mutant sus 8.5(900)-16(300)-14(1241) (defective in head fibers and the packaging ATPase) and purified as described previously (59). The packaging ATPase, gp16, was produced from the plasmid pSACB-gp16 in B. subtilis and purified by chromatography as described previously (59), or produced as a fusion protein in the SUMO Pro vector (LifeSensors, Inc) and expressed in E. coli (60). The SUMO tag was subsequently cleaved off and removed by IMAC, yielding non-tagged ATPase. A 25 kbp dsDNA packaging substrate labeled at one end with biotin was prepared by PCR, as described previously (61), from a stock solution of lambda phage DNA using primers biotin-
CTGATGAGTTCGTGTCCGTACAACTGGCGTAATC and amino-C₆-digoxigenin-
ATCCGATCTGCGTTACCGAATGGATGGATG. This construct does not contain the gp3
DNA terminal protein needed for viral assembly in vivo. The DNA was tethered to 2.8 µm
diameter streptavidin coated microspheres, and prohead-motor complexes were pre-assembled
and attached via anti-phi29 antibodies to 2.1 µm protein G coated microspheres, as described
previously (10, 13).

Packaging was initiated by bringing a microsphere carrying DNA into near contact
with a microsphere carrying prohead-motor complexes in the presence of the standard
packaging buffer containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂ with 0.5
mM ATP, as described previously (10, 13). Complexes were stalled by introducing the
standard buffer with 0.4 mM γS-ATP via a capillary tube, and were restarted by re-introducing
standard packaging buffer.

Measurements were made with a dual-trap optical tweezers system, as described
previously (10, 13), at ~23°C. The tweezers were calibrated as described previously (62, 63).
The tether length was computed from the measured force vs. fractional extension relationship
and velocities were calculated by linear fits to DNA tether length vs. time in a 3-s sliding
window. The index of dispersion in normalized velocity for each ensemble was calculated in
2.5% filling bins by taking the variance of each bin divided by its mean. The % change in
instantaneous velocity after stalling was calculated as \((v_{poststall} - v_{prestall})/v_{prestall}\), with \(v_{prestall}\) and
\(v_{poststall}\) determined from the last and first points, respectively, of linear trend lines fits to the 25
velocity data points before and after each stalling event, as shown in Fig1C of the main text.
All error bars were determined by bootstrap analysis.

Pauses in which the motor temporarily stops translocating DNA for ~1-10 s are often
observed (9). To score pausing events automatically we employed a residence time histogram
method similar to that described previously (33). Histograms of residence time vs. tether
length packaged in bins of 5% of the genome length were constructed for each packaging record and pauses were identified as peaks in these histograms exceeding a threshold determined from an equally sized ensemble of simulated packaging events not containing pauses. The median residence time within each bin was used for comparison to simulated data. Simulated packaging traces were generated according to the model described in Ref. (33), with added instrument noise as measured experimentally. The average duration and frequency of pauses as a function of the % of the genome length packaged for each ensemble were calculated in 5% genome length bins.

Chapter 4, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in PNAS. (Z. T. Berndsen, N. Keller, S. Grimes, P. J. Jardine, D. E. Smith. (2014). Nonequilibrium dynamics and ultraslow relaxation of confined DNA during viral packaging, Proceedings of the National Academy of Science.
Figure 4.1 Motor Stall-restart Experiments. (A) Schematic diagram illustrating the experimental method. Packaging is initiated and followed to ~75% prohead filling under a low applied force (5 pN) (steps 1-3). The motor is then stalled by locally injecting the non-hydrolyzable analog γS-ATP via a micro-capillary tube (step 4). After a variable waiting time packaging is restarted by reintroducing ATP (step 5). (B) Example packaging trajectory before (red) and after (blue) an imposed stall of 11.6 minutes. The grey plot shows the same data as in blue shifted back to the time of the stall, showing the acceleration. (C) Packaging rate vs. length of DNA packaged before (red) and after (blue) stalling for the event shown in (B). Black lines are linear trend lines fit to data before and after the stall point. (D) Mean change in packaging rate (blue) and motor velocity (red, rate not including pauses and slips) after short stalls (<3.4 min, average=1.5 min, n=50 events), medium stalls (3.4-10 min, average=5.7 min, n=50), and long stalls (>10 min, average=12.3 min, n=20). The bars marked "No Stall" indicate control experiments where the motor was not stalled (n=65). P-values for significance of differences vs. the next lowest stall time are indicated above the bars, and P-values for significance of differences vs. the no stall control are indicated below the bars.
Figure 4.2 Heterogeneity in Packaging Dynamics. (A) Normalized velocities measured in 3 second intervals vs. filling measured for an ensemble of packaging events (red, n=85), events with perforated proheads (blue, n=58), events measured after long stalls (green, n=20), and events with 5 mM added spermidine (grey, n = 53). The velocities are normalized by the average velocity measured with <10% genome length packaged. (B) Index of dispersion (variance divided by mean) of normalized velocity vs. filling, labeled with the same colors as in (A).
Figure 4.3 Motor Pausing Dynamics. (A) Pause duration and (B) Pause frequency per DNA length vs. filling for standard events (red), events with short stalls (light blue), medium length stalls (dark blue), and long stalls (green), and with 5 mM spermidine (grey). Inset shows frequencies of long pauses (> 5 s).
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Chapter 5

Repulsive DNA-DNA Interactions

Accelerate Viral DNA Packaging in Phage Phi29
Abstract

We use optical tweezers to study the effect of attractive versus repulsive DNA-DNA interactions on motor-driven viral packaging. Screening of repulsive interactions accelerates packaging, but induction of attractive interactions by spermidine$^{3+}$ causes heterogeneous dynamics. Acceleration is observed in a fraction of complexes, but most exhibit slowing and stalling, suggesting that attractive interactions promote nonequilibrium DNA conformations that impede the motor. Thus, repulsive interactions facilitate packaging despite increasing the energy of the theoretical optimum spooled DNA conformation.

Introduction

In the assembly of many viruses, molecular motors provide the driving forces to package DNA to near crystalline densities inside preassembled viral proheads [1-5]. The DNA, a negatively charged, semi-flexible polymer, is compacted to such high density that its bending rigidity, electrostatic self-repulsion, and entropy loss present a strong barrier to confinement [6-15]. Measurements show that viral molecular motors can exert high forces (>60 pN) and translocate DNA at rates ranging from ~100-2000 basepairs/sec [1,7,16,17]. The rate of packaging decreases with increasing prohead filling due, in part, to the buildup of large forces resisting DNA confinement [18,19]. In addition to being of biological interest, viral DNA packaging is a unique model for investigating and understanding the behavior of polymers under nanoscale confinement, which has long been a topic of interest in polymer physics [20,21].

Positively charged polyamines such as spermidine$^{3+}$, present in the cells viruses infect, are thought to play an important role in packaging by screening the DNA charge [12,22,23]. Above a critical concentration polyamines can induce a phase transition where the DNA-DNA interaction changes from purely repulsive to partly attractive, which causes free DNA in
solution to condense into a tight spool, a conformation similar to that proposed to occur in viruses [6,10,12,23-27].

The effect of repulsive vs. attractive DNA-DNA interactions has been considered in many theoretical studies and all have predicted that attractive interactions would facilitate DNA packaging by reducing the forces resisting DNA confinement. In Brownian dynamics simulations, Kindt et al. predicted arrangement of the DNA into a toroid-shaped spool that evolved into an inverse coaxial spool [8]. With a purely repulsive interaction, a more disordered structure and higher resistance forces were predicted, although it was hypothesized that the DNA would eventually equilibrate to a spool conformation. Kindt et al., Tzili et al., and Purohit et al. developed continuum-mechanics theories that assume the DNA is packaged into a minimum-energy spool conformation with local hexagonal packing [8,10-12]. These models reproduce many of the experimental trends in DNA packaging and ejection, including the sharp increase in resistance towards the end of packaging. With an attractive potential, ~10× lower resistance is predicted than with a purely repulsive one [12].

In Langevin dynamics simulations with an attractive potential, Forrey and Muthukumar predicted increased DNA ordering and structures resembling a folded toroid [14]. They also predicted that nonequilibrium dynamics would cause heterogeneity in the DNA conformations and packaging forces. Similar effects were predicted with purely repulsive interactions [13-15]. In molecular dynamics simulations, Petrov and Harvey predicted sharply reduced packaging forces with attractive interactions and toroidal DNA conformations with a central void [28]. In Monte Carlo simulations, Comolli et al. predicted heterogeneous conformations with DNA occupying the entire prohead with uniform average density [29].
Here, we report experimental studies of the effect of attractive vs. repulsive DNA-DNA interactions on viral DNA packaging dynamics in bacteriophage phi29, in which a 19.3 kbp genome (6.6 µm) is translocated into a 54×42 nm prohead [5]. We use optical tweezers to directly measure packaging of single DNA molecules into single phi29 proheads in real time in three different conditions: (1) a standard packaging buffer in which the DNA is screened mainly by Na⁺ and Mg²⁺ ions (purely repulsive regime); (2) a low concentration of spermidine, in which the repulsion is nearly maximally screened; and (3) a high concentration of spermidine, in which the interaction becomes partly attractive. We find that screened repulsive DNA-DNA interactions facilitate efficient viral packaging despite increasing the energy of the close-packed DNA conformation.

Results

Proheads were prepared as described previously and recombinant motor protein, gp16, was prepared in *E. Coli*, using a SUMO tag [30,31]. We used a 25,339 bp dsDNA construct biotin-labeled at one end prepared by PCR from φ29-DNA with no terminal proteins [32]. Measurements were made at ~23°C in the standard condition (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 0.05 mg/ml BSA, and 0.5 mM ATP), and with added spermidine trihydrochloride.

We determined that the threshold for DNA condensation was ~1 mM spermidine by conducting DNA force-extension measurements. We chose a "low spermidine" concentration of 0.8 mM, yielding purely repulsive interactions with near maximum screening, indicated by the force increasing monotonically with extension [26]. We chose a "high spermidine" concentration of 5 mM, where we observed non-monotonic, “stick-slip” force-extension behavior, indicating attractive DNA-DNA interactions [26]. A similar spermidine concentration is reported in host cells (~10 mM) [24], although the amount bound to viral
DNA in vivo is unknown and depends on partitioning in the cell and binding of other ions and proteins.

Measurements of DNA packaging were made using optical tweezers techniques similar to those described previously [18,19,33,34]. These techniques provide details on the packaging dynamics not available from bulk assays and permit measurements in condensing conditions, which are problematic in bulk due to DNA aggregation and interference with DNA digestion by DNAse. Briefly, prohead-motor complexes are attached to one microsphere and DNA molecules are attached to a second microsphere. The DNA molecule is brought into the reaction buffer containing ATP and spermidine, and then, within a few seconds, is brought into contact with the motor to initiate packaging (Fig. 1A). We note that the prohead is permeable to water and small ions including spermidine [35,36]. Measurements in standard condition and with low spermidine (maximum screening) with a small (5 pN) applied force showed fairly uniform packaging trajectories (Fig. 1B). However, with low spermidine the average packaging rate and motor velocity (translocation rate not including pauses and slips, as discussed previously [7,18,19]) are higher than in the standard condition across the whole range of filling levels (Fig. 2A&B).

The motor velocity in low spermidine also exhibits a plateau up to ~30% filling (Fig. 2B). In this low-filling regime, additional measurements show that the velocity decreases with increasing applied force (Fig. 3A). Together these measurements indicate that there is almost no resistance to packaging in this regime, because no slowing was observed. The higher motor velocity measured with low spermidine (Fig. 2B) is thus not due to a reduced resistance to packaging, but rather indicates that spermidine acts directly on the motor to increase its velocity, presumably by directly affecting the motor protein structure and/or protein-DNA interactions [37,38].
Previous studies employed the measured velocity vs. applied force relationship to infer an effective "internal force" resisting packaging [7,18,19]. However, recent studies indicate that although both prohead filling and applied force slow the motor, they have a different influence on the motor kinetics and cannot be directly equated [39,40]. In the present paper we refer to "resistance" or "load" on the motor as any interaction between the packaged DNA and motor which slows packaging.

With low spermidine, the motor velocity remains higher as the filling increases than in the standard condition, consistent with an expected reduction in packing force due to increased screening [8,12,14,18]. Thus, low spermidine accelerates packaging by directly increasing motor velocity and decreasing the load on the motor. The latter finding is consistent with theoretical studies which predict that increased screening would facilitate packaging by reducing the energy needed for DNA confinement [10,12,14,28].

The behavior observed with an attractive interaction with high spermidine is dramatically different (Fig. 1B). Large heterogeneity is observed in the dynamics of different individual complexes, characterized by a significant increase in standard deviation of packaged DNA lengths with time (Fig. 3B). Events in which the motor stalled were often observed: 51% (N=149) of complexes stalled at <50% filling vs. only 12% (N=18) in the standard condition and 15% in low spermidine (N=37). This observed inhibition was not anticipated by any of the theoretical modeling studies.

Compared with the standard and low spermidine conditions, the average packaging rate with high spermidine is sharply reduced, except for events which reached ~80% filling (Fig. 2A). 75% of complexes stall before reaching this point (N=220). However, the 25% of complexes that reach 80% filling have a significantly higher average motor velocity across the entire range of fillings (Fig. 2B). The behavior of this "fast subset" is consistent with
theoretical predictions that attractive interactions would enhance packaging. These complexes exhibit less pausing and slipping as well as higher motor velocity. The motor velocity exhibits a similar but slightly higher velocity plateau up to ~30% filling as in the standard condition. This plateau implies there is negligible resistance up to ~30% filling and that high spermidine increases the motor velocity more than low spermidine.

However, 75% of complexes are inhibited by high spermidine and the average motor velocity for the entire ensemble with high spermidine is much lower than with low spermidine (Fig. 2B) and shows a shorter plateau up to only ~10% filling. This suggests that attractive interactions cause, on average, an increase in resistance faced by the motor even at fillings as low as 10%, contrary to predictions which assume a minimum-energy spooled DNA configuration. In addition to decreasing the motor velocity, high spermidine also causes an increase in motor pausing and slipping. There is a 12-fold increase in percent time spent paused at <50% filling (N=293 events) vs. a 1.4-fold increase with low spermidine (N=249). Slips are 3× more frequent (N=5609) and 4.7× longer on average (2480±200 bp) than in the standard condition vs. 1.2× more frequent and 1.1× longer in low spermidine (N=1914). Also 53% of slips (N=2947) occurred at <50% filling vs. 10% in the standard condition (N=98) and 11% in low spermidine (N=216). These observations show that in the presence of attractive DNA-DNA interactions, the load faced by the motor in individual proheads is widely variable and often high enough to cause stalling.

We considered whether the inhibition might be due to a direct effect of spermidine on the motor rather than an effect on the packaged DNA conformation. Two observations suggest this is not the case. First, for the subset of fast complexes, high spermidine increases the inherent motor velocity at low filling more than low spermidine (Fig. 2B). Second, we observe a small fraction of events in which packaging does not show the usual trend of slowing down
and the length of DNA translocated greatly exceeds 100% of the genome length. We interpret these events as cases where the prohead is perforated such that the DNA leaks out of the prohead and thus the motor faces no resistance. A similar effect was observed in previous studies of phage lambda packaging with proheads lacking a stabilizing protein essential in that system [16]. These events provide evidence that high spermidine does not directly inhibit the motor because they displayed higher velocity (155 bp/s average, N=32) while translocating the full genome length than in the standard condition (130 bp/s, N=35). Also, the perforated-head events (exhibiting no slowing) in high spermidine exhibit ~140-fold less-frequent pausing and ~80-fold less-frequent slipping than regular events.

Although a 5 pN force was applied to keep the DNA stretched during measurements, this is insufficient to completely prevent DNA condensation [26]. In control experiments with tethered DNA, slight shortening of the DNA extension was observed (6 bp/s on average), suggesting that small sections of the unpackaged DNA tether condense during packaging. To investigate whether such condensation interferes with translocation we conducted several experiments.

First, we performed measurements with high spermidine in which we relaxed the DNA to near-zero tension during packaging to promote condensation of external DNA (Fig. 4A). The DNA was then stretched to 5 pN, revealing shortening of the extension due to partial condensation. We found that such condensation did not significantly affect the motor as 90% (N=45) of these complexes continued packaging without interruption, indicating that the motor can usually exert sufficient force to unravel sections of condensed DNA. Second, we conducted experiments in which, upon observing stalling of a complex, we ramped the force from 5 pN to 25 pN to decondense any unpackaged DNA (Fig. 4B). This did not enable any of the stalled complexes to resume packaging (N=25). Ramping to 25 pN in the standard
conditions does not halt packaging (Fig. 3A). Third, we conducted packaging measurements with a 15 pN constant applied force, high enough to prevent DNA condensation [26]. Although this force slows the motor, it does not eliminate the increase in heterogeneity caused by spermidine (Fig. 4B, inset).

**Discussion**

Since our experiments show that the increased heterogeneity observed with high spermidine is not due to direct alteration of motor function and not due to condensation of the unpackaged DNA, we conclude that it is due to heterogeneity in the load on the motor presented by varying conformations of the packaged DNA. We interpret these findings as indicating that DNA molecules packaged in individual proheads adopt widely different nonequilibrium conformations, often including highly unfavorable jammed conformations that impede the motor. Our findings further suggest that attractive DNA-DNA interactions exaggerate the formation of these nonequilibrium conformations. A small fraction of complexes in which the DNA adopts a nearer-to-equilibrium conformation package faster than in purely repulsive conditions, but in the majority the DNA is kinetically trapped in highly unfavorable non-equilibrium conformations that stall packaging. Our results indicate that repulsive DNA-DNA interactions play an important role in facilitating viral packaging by mitigating the formation of such conformations.

Our finding of faster packaging with low spermidine is consistent with models which predict that increased screening of DNA should reduce the forces resisting packaging [6,8,10,12,14] and studies showing that osmotic pressures required to inhibit ejection are reduced in conditions with increased screening [36]. However, our findings of strong inhibition with high spermidine are different than were anticipated. Studies with condensing levels of spermine$^{4+}$ found inhibition of DNA ejection by phages T5 and lambda, whereas it is
complete in the absence of polyamines [9,36,41]. Those studies indicate that condensation reduces the ejection force, suggesting that they would decrease the force resisting packaging. This notion is consistent with our finding of accelerated packaging in a small fraction of complexes, but inconsistent with our finding that the packaging is stalled in the majority of complexes. Thus, our studies imply that DNA does not generally follow the reverse conformational trajectory during ejection that is followed during packaging.

Modeling studies that incorporated attractive DNA-DNA interactions predict more ordered structures and lower forces resisting DNA confinement. The likely explanation is that these studies have considered equilibrated DNA conformations. In continuum models, packaging is assumed to be a quasi-static process in which the DNA continually relaxes to a free-energy minimum conformation [8,10-12,42]. As discussed by Tzil et al., the forces resisting packaging may be higher than the calculated statistical-thermodynamic force due to dynamic dissipative effects [10]. In most packaging simulations the DNA is periodically equilibrated [8,14,43], subject to limits on simulation time. Several studies observed effects attributable to nonequilibrium dynamics, including heterogeneous DNA conformations, fluctuating packaging forces, pausing in packaging, and higher forces resisting packaging than driving DNA ejection [13-15]. However, none of these studies predicted strong inhibition at low filling and none specifically addressed the effect of attractive vs. repulsive interactions on nonequilibrium effects.

Recent electron microscopy studies investigated condensation of DNA in phage T5 in which Spermine$^{4+}$ was added after DNA was partly ejected [44]. Toroids with outer radii as small as ~25 nm were observed, smaller than the ~80 nm prohead diameter of T5 and ~42 nm diameter of phi29. The subset of fast complexes we observe with high spermidine may correspond to cases where the DNA is able to condense into small toroids that present little
resistance to confinement (though evidently not zero resistance, as the motor does progressively slow above ~30% filling).

A potentially related effect has been observed in simulations of DNA condensation inside proheads where DNA-DNA interactions were switched from repulsive to attractive after partial packaging. Kinetically trapped non-equilibrium conformations that do not relax to toroids were predicted even at prohead fillings as low as 30% [28]. Although such conformations were not observed in continuous packaging simulations with constant attractive interactions, related effects likely cause the inhibition we observe. Notably, studies of free DNA condensation have reported conformational changes on timescales ranging from tens of minutes to hours [45,46].

In summary, we report that low concentrations of spermidine, resulting in a maximally screened repulsive DNA-DNA interaction, accelerate DNA packaging by both stimulating the motor function directly and reducing the load on the motor that builds with increased prohead filling. In contrast, a high concentration of spermidine, which induces an attractive DNA-DNA interaction, results in highly heterogeneous dynamics. A small fraction of complexes package faster than in the purely repulsive condition, but the majority of complexes exhibit dramatic slowing, pausing, and stalling at low filling, which we attribute to formation of highly nonequilibrium DNA conformations. Our results indicate that repulsive DNA-DNA interactions play an important role in facilitating efficient viral packaging despite increasing the energy of the close-packed DNA conformation.

Chapter 5 in full is a reprint that the dissertation author was the principal researcher and author of. The material appears in Physical Review Letters. (N. Keller, D. del Toro, S. Grimes, P. Jardine, D. E. Smith. (2014). Repulsive DNA-DNA interactions accelerate viral DNA packaging in phage phi29.)
Figure 5.1 Packaging Dynamics with Spermidine\(^3+\). (A) The prohead-motor complex is attached to one optically trapped microsphere (left) and the end of the DNA is attached to a second trapped microsphere (right). (B) Typical measurements with 5 pN external load in the standard condition (black), low spermidine (blue, slightly faster), and high spermidine (red, highly heterogeneous).
Figure 5.2 Motor Velocity vs. Filling with Spermidine\textsuperscript{3+}. (A) Average packaging rate and (B) motor velocity (packaging rate not including pauses and slips) vs. prohead filling under 5 pN applied force in the standard condition (black, 3rd highest initial rate), low spermidine (blue, 2nd highest initial rate), high spermidine (red, lowest initial rate), and for the subset of complexes in high spermidine that package to > 80% filling (green, highest initial rate).
Figure 5.3 Heterogeneity in Packaging Dynamics with Spermidine$^{3+}$. (A) Average motor velocity vs. applied load for complexes that reached $>30$ pN with $<20\%$ filling in the standard condition (black, $N=113$ events, lowest rates), low spermidine (blue, $N=61$), and fast (nonstalling) complexes in high spermidine (green, $N=101$, highest rates). (b) Standard deviation in DNA length packaged by different complexes for the standard condition (black), low spermidine (blue, nearly equal to standard), and high spermidine (red, highest values) under $5$ pN load.
Figure 5.4 Control Packaging Experiments with Spermidine$^{3+}$. (A) Example where a complex was packaging under 5 pN applied load (shortening of the DNA extension; section #1), and was relaxed to bring the applied force to near zero (section 2). The DNA was then stretched again to 5 pN (#3) and found to be partly condensed (indicated by 4) (dashed line shows the expected trend for packaging in the absence of condensation). Despite condensation, packaging continued (#5). (B) Example in which motor was packaging under 5 pN load (#1) and then stalled (#2). The DNA was then stretched to 25 pN to extend any condensed segments (#3), but no recovery in packaging was observed (#4). Inset shows standard deviation in lengths measured at 15 pN in the standard condition (black, lower values) and high spermidine (red, higher values).
References


Chapter 6

Single DNA Molecule Jamming and
History-dependent Dynamics During
Motor-driven Viral Packaging
Abstract

In many viruses molecular motors forcibly pack single DNA molecules to near-crystalline density into ~50-100 nm prohead shells. Unexpectedly, we found that packaging frequently stalls in conditions that induce net attractive DNA-DNA interactions. Here, we present findings suggesting that this stalling occurs because the DNA undergoes a nonequilibrium jamming transition analogous to that observed in many soft-matter systems, such as colloidal and granular systems. Experiments in which conditions are changed during packaging to switch DNA-DNA interactions between purely repulsive and net attractive reveal strongly history-dependent dynamics. An abrupt deceleration is usually observed before stalling, indicating that a transition in DNA conformation causes an abrupt increase in resistance. Our findings suggest that the concept of jamming can be extended to a single polymer molecule. However, compared with macroscopic samples of colloidal particles, we find that single DNA molecules jam over a much larger range of densities. We attribute this difference to the nanoscale system size, consistent with theoretical predictions for jamming of attractive athermal particles.

Introduction

ATP-powered motors package DNA into viral proheads via a portal nanochannel, overcoming large forces resisting DNA confinement arising from DNA bending rigidity, electrostatic self-repulsion, and entropy loss. In addition to being of biological interest, viral packaging is an experimentally accessible model for investigating effects of spatial confinement on polymer dynamics, a topic of fundamental interest in polymer physics. Although the physics of DNA packaging has been modeled theoretically using a wide variety of analytic and simulation methods, a full understanding has remained elusive.

In aqueous solutions containing monovalent and divalent salt ions DNA-DNA self-interactions are purely repulsive. We obtain this condition here with a standard packaging
buffer containing 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl$_2$, and 0.5 mM ATP. Addition of +3 or +4 ions induces attractive DNA-DNA interactions and above a critical ion concentration, the interaction becomes net attractive, which causes condensation of DNA in solution into a densely-packed form\textsuperscript{20,21}. We obtain this condition here by adding 5 mM spermidine or 20 mM spermine to the standard packaging buffer. With 100\% of the wildtype genome length packaged, X-ray scattering measurements on phage lambda virus, which packages to similar density as the phage phi29 we study here, show that +3 or +4 ions do not induce a net attractive DNA-DNA interaction because the strands are confined to such a small spacing that they are in a repulsive portion of the distance-dependent interaction potential\textsuperscript{22}. However at lower packing density, e.g. with 78\% of the wildtype genome length packaged, the X-ray measurements show that a net attractive interaction is induced\textsuperscript{22}. A net attractive interaction is induced in our studies when we add polyamine ions because we are in this regime of lower packing density.

Addition of a low concentration of +3 ions, below the threshold for DNA condensation, speeds up packaging in phage phi29 viruses\textsuperscript{23} and increases the yield of lambda phages in an in vitro assembly assay\textsuperscript{24}, consistent with packaging being facilitated by a reduction in strength of the net repulsive DNA-DNA interaction due to increased ionic screening. However with a larger concentration of +3 ions, when the DNA-DNA interaction becomes net attractive, we found that frequent stalling of DNA packaging occurs\textsuperscript{3}. This finding was unexpected because theoretical models predicted that forces resisting DNA confinement would be greatly reduced with a net attractive interaction\textsuperscript{11-13,25,26}. We present evidence here suggesting that this stalling occurs because the DNA undergoes a nonequilibrium jamming transition.

Many soft matter systems, including colloids, granular materials, suspensions, clays, pastes, and foams exhibit nonequilibrium transitions from a fluid-like to a solid-like state\textsuperscript{4,8}. 
The concept of a jamming transition was proposed to be a universal mechanism for such transitions based on the formation of a disordered stress-bearing network of geometrically-constrained particles. Characteristic features of this transition are that it occurs above a critical packing density and/or below a critical load force, and is promoted by attractive interactions. Unjamming can be induced by application of a force that exceeds the “yield force” of the jammed material. Another characteristic feature is that these systems exhibit history-dependent dynamics or “memory effects.”

The concept of jamming has been shown to be applicable to the packing of macroscopic "granular polymer chains", governed by jamming of semi-rigid loops. Here, we provide evidence that jamming can also occur in a submicroscopic polymer chain with increasing density under confinement and that net attractive self-interactions promote this. We also present data suggesting that an unjamming transition can be induced by force or by changing the DNA-DNA interaction from net attractive to purely repulsive.

**Results**

We measure packaging of single DNA molecules into single bacteriophage phi29 proheads using optical tweezers (Fig. 1a). In the purely repulsive DNA-DNA interaction condition the 6.6 μm genome length of DNA is efficiently packaged into the ~50 nm diameter prohead in ~5 minutes, reaching a final density of ~0.5 g/ml. However, in the net attractive interaction condition, 75% ± 2.5% (mean ± standard error; n = 293) of complexes stopped abruptly before packaging 80% of the genome length, an effect we refer to as "stalling." In the purely repulsive condition stalling was only observed in 8% ± 1.5% (n = 343) of complexes. Our interpretation is that stalling occurs because the packaged DNA undergoes a jamming transition and translocation halts because the yield force of the jammed DNA exceeds the maximum force the motor can exert. This finding is consistent with theoretical
predictions and experiments on colloid systems showing that attractive interactions promote jamming\textsuperscript{5, 10}.

To investigate whether viral packaging exhibits history-dependent dynamics, a signature observed in many other systems that undergo jamming\textsuperscript{4, 6, 28}, we conducted experiments in which the solution was changed during packaging to switch the DNA-DNA interactions between purely repulsive and net attractive. First, we initiated packaging in the purely repulsive condition and proceeded to various filling levels (fraction of genome length packaged). The complex was then moved rapidly, within ~1 s, into the net attractive condition. We find that the fraction of complexes that stall per unit time after switching the condition decreases with increasing DNA length pre-packaged in the repulsive condition (Fig. 1b-d). Specifically, 41% ± 3% (n=210) of complexes stalled within one minute after switching to the net attractive condition at 20% (standard deviation, 6%) filling, versus 28% ± 5% (n=89) of complexes after switching at 49% (standard deviation, 9%), versus 8 ± 3% (n=113) of complexes after switching at 66% (standard deviation, 6%) filling. This effect results in a decrease in the heterogeneity in length DNA packaged vs. time with increasing length pre-packaged under repulsive interactions (Fig. 1e). Thus, the dynamics are indeed history-dependent. Our interpretation is that the repulsive condition promotes formation of more favorable (lower energy) packed DNA conformations that persist and influence the dynamics of rearrangement of the packaged DNA after switching to the net attractive condition. History dependence was also observed in the motor velocity at some filling levels—compared with that measured after switching at 20% filling (standard deviation, 6%), the average velocity was ~6% higher over the range from 40-50% filling after switching in this range, and ~16% higher over the range from 60-70% filling after switching in this range (Supplementary Fig. 1).
We also conducted experiments in which complexes were briefly exposed to net attractive conditions at low filling (for ~20 s at filling levels up to 26%; standard deviation, 8%) then moved to the repulsive condition (Fig. 1f). Whereas no stalling was induced during the brief exposure to net attractive conditions, 39% ± 7.6% (n = 41) of complexes stalled after a return to repulsive conditions compared with only 8% ± 1.5% (n = 343) packaged with continuously repulsive interactions. This again shows that the DNA translocation dynamics are strongly history dependent and suggests that unfavorable DNA conformations formed during early stages of packaging can influence the subsequent dynamics and promote stalling.

We next analyzed in further detail the dynamics of DNA translocation in the continuously net attractive condition. Strikingly, we find that stalling is usually preceded by an abrupt and large deceleration event (Fig. 2a&b). Examples of these events are shown in Supplementary Fig. 2. 77% ± 2.5% (n = 293) of complexes exhibited maximum decelerations greater than three standard deviations below the average at the same filling level in the repulsive condition (Fig. 2c). The sudden decreases in motor velocity indicate that the DNA has undergone an abrupt transition in conformation that causes a large increase in resistance. One possible interpretation is that, at this point, the DNA undergoes a jamming transition but that the motor can generate a force that exceeds the yield force of the DNA and rapidly induces an unjamming transition, allowing translocation to continue. Given the time resolution of our measurement (~1 s) we would not expect to observe arrest because the high rate of translocation (~50 nm/s) would rapidly increase the applied force and induce unjamming. Within this interpretation, our results would imply that the yield force of individual jammed complexes is highly variable—in some it is high enough to cause irreversible stalling while in others it is lower and the motor can induce unjamming.

Notably, both deceleration and stalling events during packaging in the net attractive condition occur over a wide range of filling levels (Fig. 2d), suggesting that the density at
which jamming occurs for individual complexes is also highly variable. However, for the ensemble of complexes the probability of both deceleration and stalling events increases with prohead filling (Fig. 2e), as expected for jamming. 88% ± 2.9% (n = 226) of the complexes exhibiting deceleration events subsequently stalled. Our interpretation is that although the DNA can be un-jammed by motor force its conformation is such that it is predisposed to jam again, ultimately stalling the motor irreversibly. This finding demonstrates another form of history-dependent behavior in which the translocation dynamics of a complex depends on its past translocation dynamics. After a deceleration event the packaging rate also remains low and does not increase back to the original value. The average motor velocity exhibited by complexes following a deceleration event is lower at every filling level compared with that of complexes that did not undergo or had not yet undergone a deceleration event (Fig. 2f). It is therefore possible that the deceleration does not represent a jamming (fluid-solid) transition followed by unjamming, but rather a transition from a fluid-like state to another more viscous fluid-like state which presents higher resistance and slows the motor.

Behavior more clearly like an unjamming (solid-to-fluid) transition was observed in a different experiment in which complexes that stalled in the net attractive condition were switched into the purely repulsive condition (Fig. 3). Restarting of DNA translocation was observed in 49% ± 5.1% (n = 96) of trials, consistent with the expectation that unjamming can be induced at constant density and constant force by reducing the strength of the attractive interactions⁴,⁵. On the other hand, roughly half of complexes did not restart, suggesting that the DNA may be trapped in an unfavorable conformation, formed in the net attractive condition, which cannot quickly relax to allow packaging to resume on the timescale of the measurement. A long relaxation time is plausible given that our previous measurements revealed DNA relaxation times as long as 10 minutes even in the purely repulsive condition³³.
Almost all of the complexes which stall then later exhibit slipping of the DNA back out of the prohead. In only 2% of packaging events in the attractive condition did data recording end before a slip was observed. The average time between stalling and slipping was similar in the continuously net attractive condition (20 ± 2 sec) and in the experiments where the condition was switched after stalling from net attractive to repulsive (17 ± 4 sec). It has been shown that proheads can be perforated by freezing and thawing such that the DNA leaks out of the prohead as it is translocated and there is no buildup of force resisting DNA confinement\textsuperscript{34}. In the net attractive condition we find that only 2% ± 2% of complexes with perforated proheads slip after 1 minute compared with 21% ± 2% for regular proheads. We therefore attribute the frequent slipping after stalling observed with regular proheads not to weakened DNA grip, but to high resistance force presented by jammed DNA that causes failure of the motor-DNA grip.

**Discussion**

The single DNA molecule jamming we provide evidence for exhibits a striking difference from jamming measured in macroscopic samples of colloidal particles in that the transition in the latter case occurs over a much smaller range of packing densities. In Ref.\textsuperscript{5} a sharp fluid-to-solid transition was measured for carbon black particles as the volume fraction was increased from ~0.05 to 0.053, whereas the individual complexes in our studies stalled at DNA volume fractions ranging from ~0.05 to 0.4. This difference is likely attributable to the much smaller size of our system. Theoretical studies of disordered systems of athermal particles predict that the range of densities over which jamming occurs increases with decreasing system size (number of particles) and that attractive interactions cause jamming at lower densities\textsuperscript{9, 10}. The colloidal samples studied in Ref.\textsuperscript{5} contained >10\textsuperscript{9} particles (volumes large enough to be measured in a rheometer), whereas the viral DNA can be modeled as a
polymer with only ~100 persistence lengths (statistically uncorrelated segments in terms of tangent vector directions in free solution)\textsuperscript{35}.

Interestingly, evidence has been presented that DNA packaged in mature lambda phages (100% wildtype genome length) undergoes a solid-to-fluid like transition at increased temperature\textsuperscript{36}. The transition was reported to occur in a narrow temperature range of a few °C in conditions with net repulsive DNA-DNA interactions. Notably, in our measurements we observe much less heterogeneity in the packaging dynamics in the repulsive condition than in the net attractive condition (Fig. 1e). Some of the evidence for the temperature-dependent transition in Ref. \textsuperscript{36} was from bulk X-ray scattering and calorimetry measurements, providing statistical averages, which may not reveal heterogeneity in individual complexes. However, spring constants of individual viruses were also measured by atomic force microscopy and there appears to be higher variability in these at a temperature slightly below the transition point\textsuperscript{36}. In another potentially related study, attractive DNA-DNA interactions were found to partly suppress ejection of DNA from phage lambda and cause larger variation in lengths of DNA remaining unejected than when ejection was suppressed by osmotic pressure in purely repulsive conditions\textsuperscript{37}. The conclusion of this study that attractive interactions promotes the formation of nonequilibrium DNA conformations that effect DNA ejection is consistent with our conclusion that attractive interactions promotes formation of nonequilibrium conformations that affect the packaging dynamics.

Our findings suggest that at a particular filling level some viral proheads contain jammed DNA arrangements while others do not. At the ensemble level, the probability of jamming increases only gradually with packing density, not sharply as in macroscopic samples. Such behavior is generally consistent with the theoretical prediction that nanoscale systems should exhibit less-sharp phase transitions than macroscopic systems\textsuperscript{38}.

**Supplementary Information**
**Materials and Methods**

Measurements were made with a dual optical tweezers system as described previously\(^1\)\(^-\)\(^5\). The tension in the DNA was maintained at 5 pN by using a force feedback system that controls the trap separation. Proheads and recombinant motor protein, gp16, were prepared as described previously\(^8\). We used a 25,339 bp dsDNA construct, biotin labeled at one end, prepared by polymerase chain reaction using phage \(\lambda\) DNA as a template, as described previously\(^8\),\(^9\).

We calculated the packaging rate vs. filling for each single packaging event by linear fitting of the length packaged vs. time data in a 1.5 s sliding window. "Motor velocity" is defined as packaging rate calculated after detected pauses and slips were removed. The velocity vs. filling traces were then grouped together by filling in 5% filling bins and the reported errors in the velocities are standard errors in the means (standard deviation divided square root of the number of complexes) in each bin. Pauses were scored when the velocity in the 1.5 s window was indistinguishable from zero within the measurement resolution (translocation <6 nm) and slips were scored when the translocation in 1.5 s was <6 nm. A "stall" event was defined to have occurred at filling levels <80% if the packaging rate was indistinguishable from zero during the last 4 s of the measurement. Standard errors in reported percentages of complexes were calculated as the standard deviation of the binomial distribution \(\sqrt{p(1-p)/N}\), where \(p\) is the fraction of complexes and \(N\) the number of complexes.

All measurements in the repulsive DNA-DNA interaction condition were made at \(\sim\)23°C in a solution containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl\(_2\), 0.5 mM ATP. Measurements in the attractive condition were made in the same solution supplemented with polyamines (20 mM spermine tetrahydrochloride (Sigma \#85605) (Figs. 1 & 3) or 5 mM spermidine trihydrochloride (Sigma \#85578) (Fig. 2)). That these concentrations are above the
threshold needed to induce attractive DNA-DNA interactions was confirmed by conducting force-extension measurements of DNA molecules tethered between two microspheres to detect DNA condensation\textsuperscript{6,7}, as described previously\textsuperscript{3}. Exchange of complexes into (or out of) the solution containing polyamines was achieved by moving the complex towards (or away from) a micro-capillary tube from which the polyamine containing solution was gently flowing.

Chapter 6, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in Nature Physics. (N.Keller, S. Grimes, P. Jardine, D. E. Smith) (2016). Single DNA molecule jamming and history-dependent dynamics during motor-driven viral packaging
Figure 6.1 History Dependent Dynamics in Viral DNA Packaging. Viral DNA packaging exhibits history-dependent dynamics. (A) Prohead-motor complexes are attached to one trapped microsphere and DNA is attached to a second trapped microsphere. Packaging is initiated by bringing the two microspheres into proximity, whereupon the motor translocates the DNA. (B-D) Typical measurements of complexes that were initiated in the repulsive DNA-DNA interaction condition (blue lines) and allowed to proceed to ~20% (B), ~48% (C), or ~66% filling (D) prior to moving the complexes to the net attractive condition (red lines). (E) Standard deviation in length of DNA packaged vs. time for complexes measured in the repulsive condition (blue), net attractive condition (red), and after packaging ~20% (magenta), ~48% (green), or ~66% (grey) in the repulsive condition prior to moving them to the net attractive condition. Error bars indicate standard errors in the means calculated by applying the bootstrap method to the ensemble of datasets recorded on different complexes. (F) Typical measurements of complexes that were exposed to the net attractive condition (red) at low filling and subsequently moved to the repulsive condition (blue).
Figure 6.2 Packaged DNA Undergoing a Jamming Transition. Deceleration and stalling events. (A) Typical measurements showing abrupt decelerations and stalling of DNA translocation measured in the continuously net attractive condition (red), not observed in the continuously repulsive condition (blue). (B) Examples of velocity changes calculated in a 1 s window during deceleration events. (C) Maximum decelerations before 80% filling calculated in a 10 s window for the repulsive (bottom/blue) and net attractive condition (top/red). (D) Histogram of filling levels at which complexes exhibited deceleration events (top) and stalling events (bottom) when packaging in the continuously net attractive condition. (E) Probability, $p$, of a deceleration event vs. filling (dashed line) and probability of a stalling event vs. filling (solid line), calculated as the number of complexes that exhibit an event in each filling range divided by the number, $N$, that package to or through that range before stalling. Error bars indicate standard errors in the means, calculated as the standard deviation of the binomial distribution $\sqrt{p(1-p)/N}$. (F) Mean motor velocity vs. filling for all sections of packaging before a deceleration event (including complexes that did not exhibit a deceleration event) (dashed line). Mean velocity vs. filling for all sections of packaging after a deceleration event (solid line). Error bars indicate standard errors in the means, computed as standard deviation divided by square root of the number of complexes.
Figure 6.3 Switching the DNA Interaction During Packaging. Rescue of stalled complexes. Typical measurements in which complexes that stalled in the net attractive condition (red) were rapidly moved back to the repulsive condition (blue) causing translocation to restart. Inset: Zoomed-in plot showing one example where a stalled complex suddenly restarted (dashed lines indicate period when the complex was moved).
Figure 6.4 Motor Velocity vs. Filling. Effect of pre-packaging in the repulsive condition on motor velocity after moving complexes into the attractive condition. Average motor velocity vs filling for the complexes that first packaged to 20% filling (standard deviation, 6%) (magenta), 49% filling (standard deviation, 9%) (green), and 66% filling (standard deviation, 6%) (grey) in the repulsive condition and then proceeded to package in the attractive condition. Also shown are complexes that packaged continuously in the repulsive condition (blue) and in the net attractive condition (red). Error bars indicate standard errors in the means, computed as standard deviation divided by square root of the number of complexes.
Figure 6.5 Examples Traces of Deceleration and Stalling. Examples of deceleration and stalling events. Sections of packaging are labeled in black, deceleration events are indicated by the green arrows, and stalling events are indicated by the orange arrows. Note that slips (decreases in length of DNA packaged) almost always occur after stalling.
References


Chapter 7

Effect of DNA Screening by Cations
on the Force Driving Viral DNA Ejection
Abstract

Viral DNA ejection is a critical process in the lytic cycle of many DNA viruses. The packaged DNA, which has been compacted to near crystalline density by an ATP-powered molecular motor, is rapidly ejected out of the capsid and into a host cell. Ejection is driven by the large negative difference in free energy between DNA in solution and the fully packaged DNA. Gelbart and co-workers have shown that ejection can be partly or wholly suppressed by applied osmotic pressure [1]. They have used this osmotic suppression technique to estimate the ejection force in phage lambda. This technique has been applied to study phage T5, phage SPP1, and herpesvirus but not phage phi29, which is an important model system because the physics of tight DNA confinement has been extensively studied [2-7]. We have developed a method to adapt this technique to assess the forces driving ejection in phage phi29. Using polyethylene glycol (PEG) as the osmotic suppressor, we measure ejection in three different ionic conditions: a high Sodium⁺ condition where, in solution, the negatively charged DNA phosphate backbone is predicted to be neutralized by 80%, a high Magnesium²⁺ condition where DNA is neutralized by 88% and the standard packaging condition where the DNA is neutralized by 85%. We find that the ejection force increases with decreased DNA screening, consistent with theoretical predictions and recent packaging measurements. Ejection is inhibited in the presence of no PEG and a Spermine⁴⁺ concentration that sufficient to induce DNA condensation in solution. 60% (11.7 kb) of the genome is retained inside the phage procapsid after ejection. High Spermine⁴⁺ was also observed to inhibit ejection in bacteriophages lambda and T5 [1,8,9]. We also compare our ejection data to a continuum mechanical model that assumes that ejection and packaging are quasi-static processes and uses analytical theory to predict the ejection or packaging force as a function of DNA filling [10]. We find agreement with this model suggesting that it does a reasonable job estimating the internal force despite neglecting non-equilibrium effects. It should be noted that we are still
working on a journal paper describing these findings, so the conclusions and figures in this chapter are to be considered preliminary.

**Introduction**

Two fundamental processes in the life cycle of viruses are DNA packaging and DNA ejection (Figure 1). During packaging, the DNA is compacted to nearly crystalline density by a powerful, ATP driven molecular motor. During ejection, the fully packaged virus attaches itself to the membrane of a host cell and injects its DNA into the cell cytoplasm. In vivo ejection is triggered by the binding of a host receptor protein to the tip of the tail tube of a fully assembled dsDNA phage [11]. The packaged DNA is rapidly ejected out of the prohead through the portal channel, which has a diameter slightly less than twice the diameter of DNA in solution [12]. Ejection is driven by the large negative free energy difference between DNA in solution and packaged DNA [13].

Gelbart and co-workers have developed an osmotic suppression technique for measuring the pressure that drives DNA ejection in vitro [1]. They initiate ejection by addition of solubilized receptor protein in the presence of polyethylene glycol (PEG 8000), which is a large molecule that cannot permeate through the prohead wall. It acts as an ejection suppression agent by exerting an osmotic pressure that opposes ejection and ejection suppression increases with increasing PEG concentration. One point of view is that the ejecting DNA has to do work to displace a volume of PEG from the volume it occupies after being ejected (Figure 2) [14]. At equilibrium with a certain length ejected, the force resisting ejection equals the force driving ejection. In lambda, where ejection is conducted under high Magnesium\(^{2+}\) conditions (1x TM or 50 mM TrisHCl, 10 mM MgSO\(_4\), pH 8.0) and at 37 °C, the fraction of DNA ejected monotonically decreases with increasing added PEG, up to a point where complete suppression is achieved. The pressure required to fully suppress ejection in lambda is ~25 atmospheres (atm) [1,15].
This osmotic suppression technique has been used to measure ejection pressures in phage lambda, phage SPP1, herpesvirus, and phage T5 [16-18]. In all these systems the pressures needed to suppress ejection differed and ranged between 10-30 atm [16-18]. A notable and unique result occurred in bacteriophage T5. Multiple un-ejected lengths were observed at low pressures [17]. The authors argued that these multiple un-ejected lengths are due to non-equilibrium conformational states of the internal DNA during ejection.

We recently discovered that prohead filling slows the motor by loading the motor and reducing the ATP binding rate, which we argued is an allostERIC mechanism [9]. Before 75% filling, the deceleration in packaging rate is entirely due to this allosteric mechanism. After 75% filling, both the allosteric mechanism and the internal force slow the motor. The internal force begins to rise at 75% filling and reaches a final value of ~22 pN at 100% filling [7,19]. The Bustamante lab at UC Berkeley has also measured the packaging force using optical tweezers [19]. They measure a dependence of packaging force on filling that is similar to what we measure.

Compared to other phage systems, phi29 is unique in many ways. Phi29 is the only system where we have experimental estimates of the forces resisting packaging. Comparing the magnitudes of the ejection force against the packaging force answers many fundamental questions on the energetics of packaging and ejection. Many theoretical models assume that both ejection and packaging occur as quasi-static, thermodynamic processes. Under the quasi-static assumption, the ejection force with a particular length of DNA remaining inside the prohead is equal to the force resisting packaging with the same length packaged. However, some simulation studies predict that non-equilibrium dynamics during packaging would cause the ejection force to be lower than the packaging force [20]. We recently showed that the packaged DNA exhibits non-equilibrium dynamics (Ch. 4), through observing motor velocity changes following DNA relaxation, but the changes in velocity were only ~20% and because
we were at ~75% filling they are, in large part, attributable to the allosteric regulation mechanism discussed in Ch. 8. Thus, we could not conclude anything regarding the extent to which non-equilibrium effects contribute to the internal force at higher filling levels. In this study, we attempt to address whether packaging and ejection are affected by non-equilibrium dynamics by comparing our ejection data to our packaging data, and compare both against a continuum-mechanical model that neglects non-equilibrium affects.

We conducted ejection measurements with phage phi29 using PEG as the osmolyte (Figure 2). We also studied the effect of added Spermine4+ in the absence of PEG. The dimensions of the prohead are 42 x 54 nm and the length of the genome is 6.6 µm. Proheads, gp16, and phi29 DNA were prepared as described previously [5,21]. To carry out the ejection procedure, we first packaged the proheads in bulk in the high Sodium+, high Magnesium2+, or standard condition (0.5x TMS). The bulk packaging assay for phi29 was pioneered by the Dwight Anderson Lab [21,22]. We add proheads, motor protein (gp16), and phi29 DNA and incubate this mixture for 5 min to allow the prohead-motor complexes to form. To initiate packaging, we add ATP and incubate this mixture for 15 minutes to allo allow packaging to complete. To digest any un-packaged DNA, we add 2 units of DNAse I (NEB, Inc.) and incubate this mixture for 10 minutes at room temperature.

We triggered ejection in the same ionic condition that we initiated packaging in. We either added PEG or Spermine4+ to the packaged proheads. Volumes of PEG were added from a 50% weight by weight (w/w) stock solution that was prepared by mixing 10 grams of PEG 8000 with 10 grams of water. For ejection with Spermine4+, we added a 2 µl aliquot from a 50 mM Spermine4+ stock solution such that the final Spermine4+ concentration during ejection was 10 mM. In order to eject the DNA after packaging we found that heating the samples to 50 °C causes a fraction of the fully packaged complexes to eject their entire genome. We use this technique because it allows us to control conditions during packaging, but also because
the receptor needed to trigger ejection from assembled phi29 phages is unknown. Any segment of the packaged DNA that is ejected out of the capsid is digested by DNase I. We confirmed that DNase I digests DNA in the presence of PEG or Spermine$^{4+}$. After incubating the packaged proheads with added PEG or Spermine$^{4+}$ at 50 °C for 30 minutes, we ruptured them by adding EDTA and proteinase K, and incubated the mixture at 65 °C for 30 minutes. This treatment releases the packaged DNA and inhibits further DNA digestion by DNase I.

We extracted the DNA retained inside the prohead after ejection by first treating the sample with an equal volume of phenol chloroform isoamyl alcohol (25:24:1 ratio) at pH 7.5. After centrifuging this mixture for 5 minutes at 13,000 g (g = gravitational acceleration = 9.8 m/s$^2$) at room temperature, we pipetted the upper volume, which now no longer contains PEG and proteins, into a clean test tube. To this test tube, we also added 1 uL of 20 mg/mL of glycogen and 2.5 volumes of 100% ethanol and incubated this sample overnight at -20 °C. This treatment precipitates the DNA into a white pellet that can be easily seen by eye. We performed two more washes with 70% ethanol and let the pellet air dry at room temperature for 15 minutes. This method for extracting DNA from packaged phi29 proheads follows an established procedure [23]. We then added 20 uL of TE (50 mM TrisHCl pH 8.0, 1 mM EDTA) and quantified the length of DNA by applying standard electrophoresis.

**Results**

We conducted ejection measurements at PEG concentrations that ranged between 0% and 35% (w/v) in three different ionic conditions: a high Sodium$^+$ condition (100 mM NaCl, 1 mM MgCl$_2$, 25 mM TrisHCl pH 7.5), a high Magnesium$^{2+}$ condition (1xTM = 50 mM TrisHCl, 10 mM MgSO$_4$, pH 8.0) and the standard packaging solution (0.5x TMS = 50 mM NaCl, 5 mM MgCl$_2$, 25 mM TrisHCl, pH 7.5) (Figure 3 and 4). As mentioned above, we packaged in the same ionic condition that we ejected in. For example, to measure ejection in the high Sodium$^+$ buffer, we first packaged in high Sodium$^+$ and then ejected in high Sodium$^+$. 
Sodium$^+$ and Magnesium$^{2+}$ ions compete with each other for binding onto the negatively charged DNA phosphate backbone and increase the net DNA charge [24]. In the high Sodium$^+$ condition, theory predicts that, in solution, \(~80\%\) of the negatively charged DNA phosphate backbone is neutralized by mostly Sodium$^+$ ions. In the high Magnesium$^{2+}$ condition, \(~89\%\) of the DNA backbone is neutralized by mostly Magnesium$^{2+}$ ions. In 0.5x TMS, \(~85\%\) of the DNA backbone is neutralized by both Sodium$^+$ and Magnesium$^{2+}$ ions [3]. To convert PEG concentration to osmotic pressure, we used an empirical relationship described in Reference 5. The osmotic pressure \((P)\) is
\[
P = -1.29G^2T + 140G^2 + 4G,
\]
where \(T\) is the temperature in Celsius and \(G\) is quantity that expresses the PEG concentration in normalized form \((G = w/(100 - w)\) where \(w = M_{PEG}/M_{solution}\)). The density of our stock PEG solution (50% w/v) is 1.09 g/mL.

At this current stage of the project, we are unable to fully suppress ejection. Time permitting, we may try preparing a 60% (w/w) PEG stock solution to carry out ejection measurements at an osmotic pressure larger than 30 atm to see if we are able to completely suppress ejection. On the other hand, such a solution is difficult to pipette due to its high viscosity. We are also unable to resolve DNA fragments with lengths larger than 16 kb using standard gel electrophoresis. To resolve larger DNA fragments, we would need to apply pulsed field gel electrophoresis. We are currently in the process of building an asymmetric pulsed field inversion system.

We find that the fraction of DNA ejected increases with decreased DNA screening at a given osmotic pressure (Figures 3, 4, and 5). For example, at an applied osmotic pressure of \(~12\) atm, \(~50\%\) of the genome is ejected in the high sodium condition, \(~38\%\) of the genome is ejected in the standard condition and \(~30\%\) is ejected in the high magnesium condition (Figure 5). In simple models the relationship between the ejection force and osmotic pressure is linear,
\[
F = P\pi R_{DNA}^2
\]
where \(P\) is the applied osmotic pressure, \(F\) is the ejection force and \(R_{DNA}\) is the
effective radius of the DNA [15]. For example, a 10 atm increase in osmotic pressure is equivalent to a 4.5 pN increase in ejection force. Thus, our finding that the fraction of DNA ejected increases with decreased DNA screening implies that the ejection force increases with decreased DNA screening.

Our finding that the ejection force increases with decreased DNA screening is in agreement with theoretical predictions on ejection [3,10,13,25,26]. Analytical theory predicts that the force due to the interstrand repulsion energy is the largest contributor to the total ejection force. This distance-dependent interaction energy is estimated empirically via experiments in which short DNA segments are condensed by an applied osmotic pressure. Since the electrostatic repulsion energy is increased with reduced DNA screening, the ejection force is predicted to increase with decreased DNA screening [3, 11-12, 19]. It has been pointed out that, in addition to electrostatic repulsion, this empirical interaction potential would include entropic effects as well [27,28]. It is generally thought that conformational entropy of the DNA chain would decrease with increased packaging due to higher ordering and constrained degrees of freedom, but there may also be significant entropic effects relating to hydration of the DNA (arrangement of the water molecules) which are difficult to predict.

Our lab’s previous experimental study of the dependence of packaging rate on ionic conditions also agrees with our ejection findings [16]. Reference 16 conducted packaging measurements with optical tweezers in almost identical ionic conditions. Packaging with high Magnesium^{2+} was found to enhance packaging because the packaging rate was constant up to 30% filling indicating that the resistance to packaging due to the packaged DNA is negligible in this filling window. In contrast, the packaging rate immediately drops after 5% filling in 0.5x TMS indicating that the packaged DNA immediately presents resistance to the motor. Packaging with high Sodium^{+} was found to inhibit packaging because the deceleration in packaging rate is larger than with 0.5x TMS. Our ejection results and the results on packaging
from Reference 16 suggest that increased DNA screening by cations lowers the total free energy of the packaged DNA and lowers the ejection and packaging forces.

Between an osmotic pressure of 1 and 3 atm, we occasionally observe two un-ejected DNA fragments (Fig 3 and Fig 4) in all three ionic conditions. The mass of the smaller fragment is always larger than the mass of the longer DNA fragment. The length of the longer fragment remains fixed at a given pressure and increases as the pressure is increased from 1 to 3 atm. As mentioned in the introduction, studies with bacteriophage T5 also observed multiple, non-ejected lengths at low pressures and attributed it to the formation of non-equilibrium DNA conformations during ejection [18]. The same process could also be occurring in our ejection measurements with phi29. We showed that the relaxation time of the packaged DNA at 75% filling is at least 10 minutes suggesting that the relaxation time of fully packaged DNA is significantly longer than 10 minutes [5]. The fully packaged genome in our ejection measurements is not in its equilibrium conformation because the amount of time that we wait between triggering ejection and initiating packaging is only ~10 minutes. Our interpretation is that the larger non-ejected length is due to a non-equilibrium conformation of the packaged DNA. The packaged DNA could undergo non-equilibrium dynamics during ejection due to either the packaged DNA adopting a non-equilibrium conformation during packaging, the packaged DNA adopting a non-equilibrium conformation during ejection or a combination of both processes. We use the smaller DNA fragment for our calculations because since it is the fragment with the highest mass and its size increases with applied osmotic pressure it is reasonable to assume that it corresponds to the DNA retained under equilibrium conditions.

Ejection is partially suppressed in the presence of a Spermine⁴⁺ concentration that is sufficient to induce DNA condensation. The DNA-DNA interaction when the DNA is condensed is partly attractive (when there is sufficiently large spacing between the strands; at
close spacings the interaction is still expected to be repulsive). The fraction of the genome that is retained after ejection is 60.6 % (11.7 kbp) +/- 0.1 % (Fig 6). Spermine$^{4+}$ also partially suppresses ejection in phage lambda where the fraction of the genome retained after ejection is also ~60% (29.1 kbp) [9,29], consistent with expectations since the two viruses have approximately the same DNA packing density with 100% of the genome length packaged. The authors of that study suggested that the reason ejection is suppressed after 40% of the genome has been ejected is that the remaining 60% of the DNA forms a condensed toroid inside the prohead, as occurs with free DNA in solution. The confinement due to the prohead no longer affects the DNA conformation because the radius of the toroid is smaller than the effective average radius of the prohead. They propose that since the packaged DNA forms a toroid with or without being confined inside the prohead, the change in free energy is zero resulting in a zero ejection force. Spermine$^{4+}$ was also found to partially inhibit ejection in phage T5 whose genomic length is 114 kbp. However, unlike phi29 and lambda where there is only a single un-ejected DNA fragment, in T5 three non-ejected DNA fragments (35 kbp, 46 kbp, and 100 kbp) were observed, which the authors attribute to non-equilibrium dynamics [8].

We compared our ejection data to a continuum mechanical model that predicts the packaging or ejection force as a function of DNA filling. The model was developed by the Rob Philips lab, which built on models developed by the Gelbart and Ben-Shaul groups, and assumes that the forces driving ejection are due to bending energy and electrostatic repulsion among neighboring DNA strands [10]. In conditions where the DNA-DNA electrostatic interaction energy is purely repulsive, the empirically determined intrastrand energy ($E$) is equal to $E = L\sqrt{3}F_o(c^2 + cd)e^{-d/c}$, where $L$ is DNA packaged (nm), $d$ is the distance between adjacent DNA strands (nm), and $c$ and $F_o$ are parameters that characterize the strength of the electrostatic interaction. We predicted the ejection force as a function of DNA filling using this model and by making the following assumptions. For simplicity we assume the
shape of the phi29 capsid, which is in reality slightly prolate, is a sphere with a radius of 21 nm (such that it matches the phi29 capsid volume). The constants in the electrostatic energy, $c$ and $F_o$, were set to 0.27 nm and 55 pN/nm$^2$, respectively based on values determined from reported X-ray scattering measurements on condensed DNA solutions that used PEG as the condensing agent in an ionic condition that is similar to ours [30]. Our measured ejection force in 0.5x TMS is in agreement with the prediction from the mechanical model (Figure 7A). The pressure required for full suppression, which is computed by extrapolating to 0% filling, is ~60 atm.

Assuming quasi-static equilibrium during packaging and ejection, the ejection force is equal to the packaging force but with a simple coordinate transformation (DNA ejected (%) → 100% – DNA packaged (%)). We proceeded with this transformation to predict the packaging force in 0.5x TMS using the mechanical model. There is agreement between our measured packaging force data and our predicted packaging force (Fig 7B) [7]. The measured packaging force from the Bustamante Lab inferred in an independent manner is also shown in Figure 7B and also shows good agreement [19]. Thus, the “inverse spool” model developed by Gelbart, Ben-Shaul and coworkers, and Phillips and coworkers is a good approximation to the ejection and packaging process in viruses.

The fact that our data are well fitted by a mechanical model answers many questions on whether the packaging and ejection force are affected by non-equilibrium dynamics. We recently discovered that a relaxed DNA conformation exhibits an increased motor velocity [5]. Our measurements though were conducted at 75% filling where the internal force is nearly zero. We could only conclude that the allosteric mechanism increased the velocity by affecting the motor-ATP interaction because it “sensed” a relaxed DNA conformation [7]. It remains unknown if the internal force has a non-equilibrium component that is large enough to noticeably slow the motor. Our data suggest that the forces in ejection and packaging are not
largely affected by non-equilibrium dynamics because both sets of data agree with a model that neglects non-equilibrium dynamics. This conclusion also indicates that the ejection process and packaging process are driven by forces of similar magnitude.

For future measurements, we plan on testing whether ejection with phi29 exhibits history dependent dynamics. Phi29 is the only system to our knowledge that has a robust and extremely efficient in-vitro bulk packaging assay. We have the ability to control the experimental conditions during packaging and measure their effect on subsequent ejection fairly soon after packaging is complete. Conversely, ejection assays on other phage systems such as T5, SPP1, and lambda, are done using phages that are fully assembled in vivo and extracted from infected bacteria. With these phage systems, the ionic condition during packaging cannot be directly controlled. Since we have already shown that altering the ionic conditions during packaging causes history dependent-dynamics, it would be very interesting to test whether ejection is affected by the ionic condition that packaging was initiated in [31]. For example, we would package in an unfavorable condition (the high Sodium\(^+\) condition for example), and then eject in the standard packaging buffer (0.5x TMS). To see if the packaged DNA had any “memory” of being packaged in an unfavorable condition, we would compare the fraction of DNA retained after ejection against packaging and ejecting in the standard condition. We are currently carrying out experiments like this.
Figure 7.1 Phage Lifecycle. Lifecycle of a virus showing the ejection and packaging process. The viral DNA is packaged by proheads inside the cell [4]. After cell lysis, the packaged proheads attach onto the membrane of a healthy cell and inject their DNA into the cell [1]. (Note: Injection and ejection refer to the same process in the figure above).
Figure 7.2 Schematic of Ejection with Phi29. Diagram of our ejection technique. Phi29 DNA is packaged in bulk by pre-assembled prohead-motor complexes at 22 °C. Ejection is carried out by incubating the packaged proheads at 50 °C in the presence of DNase I and either PEG or Spermine$^{4+}$. DNA that is ejected is digested by DNase I. PEG increases the osmotic pressure of the ejection solution causing ejection to be suppressed. Spermine$^{4+}$ also suppresses ejection by inducing DNA condensation inside the phi29 prohead.
Figure 7.3 Measuring Un-ejected DNA Lengths with Electrophoresis. Ejection was carried out in (A) 1xTM (high Magnesium$^{2+}$ condition), (B) 0.5x TMS solution (standard condition), and high Sodium$^{+}$ solution (not shown) at increasing PEG concentrations. The fraction of DNA retained after ejection was quantified using standard agarose gel electrophoresis. The DNA retained increases with increasing PEG concentration. The second, larger DNA fragment, which is discussed below, is observed at PEG concentrations between 10% (~1 atm) and 15% (~3 atm). Its intensity is much less than the intensity of the smaller DNA fragment.
Figure 7.4 Effect of Ionic Screening on Ejection as Revealed by Electrophoresis. Ejection was carried out in a high Sodium\(^+\) solution, high Magnesium\(^{2+}\) solution, and the standard packaging solution. The osmotic pressure in (A) is \(\sim 3\) atm and in (B) is \(\sim 5\) atm. The length of DNA retained after ejection increases with increasing DNA screening. In (A), there is an intermediate DNA fragment in the high Sodium\(^+\) solution lane (3rd lane) and in the 0.5x TMS solution lane (5th lane).
Figure 7.5 Increased DNA screening Lowers Ejection Pressure. Fraction of DNA ejected versus osmotic pressure for the three different ionic conditions investigated: High Sodium\(^+\) (red), 0.5x TMS (black), and 1x TM (high Magnesium\(^{2+}\)) (blue).
Figure 7.6 Partial Suppression of Ejection in High Spermine$^{4+}$. Electrophoresis of ejection in 10 mM Spermine$^{4+}$ plus 0.5x TMS. The fraction of DNA retained with Spermine$^{4+}$ is 11.7 kb or 60% of the full genome length (19.3 kb). As a comparison, ejection with ~3 atm of osmotic pressure in 0.5x TMS is also shown.
Figure 7.7 Comparing Ejection and Packaging Forces to a Model. Comparing our ejection and packaging data to a continuum mechanical model that was developed by Reference 12. (A) Fraction of genome ejected versus osmotic pressure. The fraction of DNA ejected according to the model (red) and our ejection measurements in 0.5x TMS (blue). (B) Packaging force versus fraction of genome packaged. The packaging force according to the model (red), our packaging measurements (Reference 9) (blue) and packaging measurements from the Bustamante laboratory (Reference 10) (black).
References


Chapter 8

Continuous Allosteric Regulation of a Viral Packaging Motor by a Sensor that Detects the Density and Conformation of Packaged DNA
Abstract

We report evidence for an unconventional type of allosteric regulation of a biomotor. We show that the genome packaging motor of phage phi29 is regulated by a sensor that detects the density and conformation of the DNA packaged inside the viral capsid and slows the motor by a mechanism distinct from the effect of a direct load force on the motor. Specifically, we show that motor-ATP interactions are regulated by a signal propagated allosterically from inside the viral shell to the motor mounted on the outside. This signal continuously regulates motor speed and pausing in response to changes in either density or conformation of the packaged DNA and slows the motor prior to the buildup of large forces resisting DNA confinement. Analysis of motor slipping reveals that the force resisting packaging remains low (<1pN) until ~70% then rise sharply to ~23pN at high filling, a value several fold lower than previously estimated under the assumption that force alone slowed the motor. These findings are consistent with recent studies of the stepping kinetics of the motor. The allosteric regulatory mechanism we report allows dsDNA viruses to achieve rapid, high density packing of their genomes by limiting the buildup of nonequilibrium load forces on the motor.

Introduction

A critical step in the assembly of many dsDNA viruses is the packaging of the viral genome into preformed prohead shells (1-4). In phage phi29, a 19.3 kbp genome is packaged into a 42×54 nm prohead via a ~4 nm diameter portal channel (5). This channel is comprised of a ring of portal proteins (gene product 10, gp10, also referred to as the head-tail "connector"). A ring of the packaging motor proteins (gp16) docks to the portal on the exterior of the prohead via an intervening ring of RNA molecules (prohead RNA, pRNA) (Fig. 1). Gp16 converts chemical energy from ATP hydrolysis into mechanical work to translocate the DNA through the portal. This is a remarkable process because near-crystalline packing density
of the DNA is achieved against large resistance forces anticipated to arise from DNA bending rigidity, electrostatic self-repulsion of DNA segments, and entropy loss (6-10).

The dynamics of DNA translocation and forces generated by the molecular motor have been measured in the bacteriophage phi29, lambda, and T4 systems via single DNA molecule manipulation with optical tweezers (11-22). Two striking observations observed in both phi29 and lambda are that the motor velocity decreases sharply with increasing prohead filling and also decreases with increasing externally applied force (11, 14, 16).

Recently, we showed that the DNA inside phage phi29 undergoes nonequilibrium dynamics during packaging, which slows the motor, causes heterogeneity in packaging rates of individual viruses, and causes frequent pauses in motor translocation (23). At high prohead filling we showed that the DNA adopts a nonequilibrium conformation that relaxes on a timescale >10 minutes, which is longer than the packaging reaction (~6.6 minutes, on average). The observed heterogeneity in packaging rates indicates that the DNA packaged in different individual viruses adopts different conformations and that the motor velocity depends not only on the length of DNA packaged, but also its conformation.

We report here that the amount of DNA inside the prohead, as well as its conformation, also indirectly influence motor function in a manner that is distinct from the influence of the forces resisting DNA confinement that directly load the motor. Much of the decrease in motor velocity with filling is attributable to this effect. Since only part of the slowing can be attributed to load forces, our present findings show that the previous analysis based on an assumption that load force was the only factor slowing the motor overestimated the resisting forces (11, 13, 16). Detailed measurements of motor slipping presented here suggest the force resisting DNA confinement is quite low until ~70% filling, but then builds rapidly during the final stages of packaging. The surprising implication of these findings is that the motor, besides being directly affected by load force, is also indirectly regulated by an
allosteric interaction between the DNA packaged inside the prohead and the motor mounted on the outside. We further show that these findings are consistent with recent studies demonstrating highly coordinated burst-dwell stepping kinetics of the packaging motor (26).

Materials and Methods

Phi29 components were provided by Dr. Shelley Grimes and Dr. Paul Jardine and prepared as described previously (23). A 25 kbp dsDNA packaging substrate labeled at one end with biotin was prepared by PCR, as described previously (23, 27). The DNA was tethered to 2.1 µm diameter streptavidin coated microspheres, and prohead-motor complexes were pre-assembled and attached via anti-phi29 antibodies to 2.1 µm protein G coated microspheres, as described previously (13, 16, 23).

Packaging was initiated by bringing a microsphere carrying DNA into near contact with a microsphere carrying prohead-motor complexes in the presence of the standard packaging buffer containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2 with 0.5 mM ATP, as described previously (13, 16). Exchange between solutions containing ATP and S-ATP was achieved using a custom made microfluidic flow cell. Measurements were conducted in the center channel filled with gently flowing ATP solution. The flow cell was attached to a translation stage, allowing a tethered complex held fixed in the laboratory frame with dual tweezers to be rapidly moved near the end of a capillary tube delivering a 0.4 mM S-ATP solution in the same background buffer, causing rapid (<1 s) solution exchange and stalling the motor. The motor was then restarted by moving the complex away from the capillary tube and back into the flowing ATP solutions, again achieving nearly instant solution exchange.

Measurements were made with a dual-trap optical tweezers system, as described previously (13, 16), at ~23°C. Two measurement modes were used. In the "force-clamp" mode, the applied tension is held constant at a preset value by use of feedback control system
that adjusts the separation between the two traps. In the "fixed trap position" mode, the tension is allowed to build as packaging proceeds. The tweezers were calibrated as described previously (28, 29). The tether length was computed from the measured force vs. fractional extension relationship and all velocities were calculated by linear fits to DNA tether length vs. time in a 3-s sliding window. All error bars were determined by bootstrap analysis.

Pauses in which the motor temporarily stops translocating DNA for ~1-10 s and slips in which segments of DNA rapidly exit the prohead are often observed (11). To score pausing events automatically we employed a residence time histogram method described previously (18). Slips were scored for both fixed-force and fixed-position measurements as any increase in DNA tether length that occurred within ~1s and were larger than a threshold determined from an equally sized ensemble of simulated packaging events not containing slips. Simulated packaging traces were generated according to the model described in Ref. (18), with added instrument noise as measured experimentally. The average frequency of pauses and slips as functions of the % of the genome length packaged for each ensemble were calculated in 5% genome length bins.

**Results**

We use optical tweezers to directly measure the packaging of single DNA molecules into single phage phi29 proheads using techniques modified from those we described previously (11, 13, 16, 23). In brief, prohead-motor complexes are attached to one microsphere and DNA molecules are attached to a second microsphere. When a DNA molecule is brought into contact with the motor in the presence of ATP packaging initiates and DNA is translocated into the prohead (Fig. 2a). We continuously track the length of DNA packaged vs. time by using a feedback control system to apply a constant force of 5 pN, which is small compared with the maximum force the motor can exert (>60 pN).
The most obvious way that DNA packaged inside the prohead can influence the motor function is through forces resisting DNA translocation which directly load the motor, which we call “internal force” (11, 12, 18) (Fig. 1). These forces are expected to arise because confinement of the DNA is an energetically unfavorable process (6-10). Such internal force exerts a load on the part of the motor that grips and translocates the DNA through the portal channel and slows the motor by reducing any force-dependent rate constants in the mechanochemical cycle. In standard theoretical analyses the presence of a resisting load force $F_{\text{load}}$ during a DNA translocation step of size $\Delta x$ increases the mechanical work energy by an amount $F_{\text{load}}\Delta x$, which increases reaction energy barriers (30, 31). To probe the motor’s response we can artificially apply a load force by applying tension to the unpackaged DNA tether (Fig. 1). The measured velocity $v$ has been shown to decrease with increasing applied force $F_{\text{load}}$ in accord with the formula $v = 1/(a + b \cdot \exp(c \cdot F_{\text{load}}))$ predicted by Chemla et al., where $a$, $b$, and $c$ are constants related to kinetic model parameters described in Ref. 12. The motor also slows progressively as the prohead fills with DNA (11, 13, 16). Under an assumption that load force was the only factor slowing the motor, the above relationship was used to infer the magnitude of the internal force as the prohead filled (11, 16). Surprisingly, however, we present evidence here that load force is not the only factor influencing the motor function. We show that as the prohead fills, the building density of DNA has an additional effect on the motor that is distinct from the effect of load force and contributes significantly to the change in motor dynamics at high fillings.

Our first finding demonstrating this effect comes from measurements in which we stalled the motor with a non-hydrolyzable ATP analog ($\square$S-ATP) and restarted it by reintroducing ATP. This was done by moving the packaging complex in front of a capillary dispensing $\square$S-ATP and then moving it back into the main flow chamber containing ATP. In this manner we achieved exchange back to ATP in <1 s. Strikingly, we find that the time it
takes the motor to restart following this nucleotide exchange is strongly dependent on prohead filling. At low prohead filling the motor restarts rapidly, within a few seconds (Fig. 2b), but at high filling the restart time can increase to longer than 1 minute (Fig. 2c). The average restart time measured for an ensemble of experiments increases continuously with increasing prohead filling (Fig. 2d). This increase is not attributable to increasing load force because it has been previously shown that the dissociation rate of \(^{35}\text{S}-\text{ATP}\) measured at low filling is independent of applied force (12). Our present finding therefore implies an indirect effect of prohead filling on motor-ATP interactions.

Our second finding demonstrating this effect comes from detailed studies of pausing in DNA translocation. In measurements with low load (5 pN) we observe short pauses of typically ~1-10 seconds in DNA translocation that occur with a frequency that increases strongly with increasing filling (Fig. 3a), also observed in earlier studies (11, 23, 26). We present additional data here showing that pausing, measured at low prohead filling, is not significantly induced by increasing load (Fig. 3b). Thus, the observed increase in pausing with increasing filling cannot be attributed to buildup of force resisting DNA translocation and, rather, must be attributed to an independent effect of prohead filling on motor function.

Our third finding demonstrating this effect comes from studies of motor slipping. Slips were defined as events in which a length of DNA suddenly came backwards out of the prohead, typically ~30-150 bp. In sharp contrast to the trend observed with pausing, the frequency of slipping increases both with increasing filling (Fig. 4a) and with increasing applied load (Fig. 4b). We interpret these findings as indicating that slipping occurs when the motor loses grip on the DNA and that the probability of slipping increases with increasing load. In this case, the buildup of load force can be inferred by relating the frequency of slipping vs. load force, measured at low filling, to the frequency of slipping vs. filling (Fig.
4c). The inferred internal force is very low (<1 pN) until ~70% filling, where it then begins to builds rapidly and reaches a maximum of ~23 ± 7 pN at the end of packaging.

In earlier analyses we inferred internal force under the seemingly reasonable assumption that the decrease in motor velocity with filling was completely due to this force loading the motor (11, 13, 16). However, the magnitude of the internal force deduced under that assumption is several-fold higher than that inferred by our present measurements of slipping frequency (Fig. 4b). Most notably, the motor velocity (packaging rate not including pauses and slips) decreases by ~50% going from 0 to 70% prohead filling (Fig. 5a), but the measurements of slipping suggest that there is very little internal force (<1 pN) up to this point. Based on the measured velocity vs. load force relationship (Fig. 5b), 1 pN of internal force would only cause a ~2% reduction in velocity, which cannot explain the measured ~50% velocity decrease going from 0 to 70% filling. Thus, these findings further demonstrate that much of the slowing of the motor is not caused by load force, but again must be attributed to an indirect effect of the packaged DNA on motor function.

Discussion

An unconventional type of allosteric regulation

We refer to the reported slowing of the motor with prohead filling due to modulation of motor-ATP interactions as allosteric regulation in analogy to the classical phenomenon wherein an enzyme's activity at one site is regulated by the binding of an effector molecule at a second site. Here, the motor's DNA translocase activity, which is coupled to the ATP hydrolysis cycle, is allosterically regulated by the presence of increasing amounts of DNA packaged inside the prohead. This allosteric signal negatively regulates the packaging rate by both reducing the motor's translocation speed and increasing the frequency of pauses. This regulation likely helps to mitigate the formation of non-equilibrium DNA conformations that we have demonstrated can cause stalling and slipping of the motor (23, 32).
We note that this regulation is unlike typical allosteric regulation in two ways. First, the prohead filling sensor is non-specific in the sense that it does not appear to involve binding of a specific DNA sequence to a complementary binding site. Studies using several different DNA substrates observe motor slowing independent of the sequence being packaged (11, 13, 16, 23, 32). Rather the motor function is modulated by an increasing density of confined DNA, presumably through non-specific interactions with the portal and/or prohead wall. Second, whereas binding of an effector molecule typically causes a discrete change in enzyme activity, here the velocity of the motor complex decreases continuously with increasing length of DNA packaged. Since our experiments directly measure the rates of single motor complexes, it is clear that this continuous change is of the activity of individual motors and not simply due shifting sub-populations of complexes having two distinct activity levels (e.g., with effector bound vs. unbound).

**Structural basis for the allosteric effect**

Structural studies based on cryo-electron microscopy show that the portal channel through which the DNA is translocated extends from the interior of the prohead to the exterior where the motor protein is attached via the intervening pRNA (Fig. 1) (25, 33, 34). The force resisting DNA packaging is directly transmitted to the motor via its contacts with the section of DNA threaded through the channel (Fig. 6a). However it is quite clear that the section of DNA already packaged inside the prohead does not directly contact the motor protein and therefore cannot directly affect its operation. The packaged DNA only touches the inner wall of the prohead and a portion of the portal channel that extends into the prohead interior (25, 33, 34). Our results therefore imply that the packaged DNA must interact with the motor protein via a long distance allosteric signal (Fig. 6b). This signal must be transmitted ~100Å from the interior of the prohead to the motor on the exterior via the intervening prohead shell and/or portal protein and pRNA.
Cryo-electron microscopy studies (Cryo-EM) of several different phages including phi29 show that the packaged DNA is in contact with the portion of the portal channel exposed to the interior (25, 34-39). Two particularly striking observations are: (i) a ring of DNA appears to wrap around the portal, appearing to "squeeze" it, and (ii) the portal adopts a different conformation when incorporated in the head than is observed for isolated portals (37, 38). Because the portal was only imaged at one filling level (fully packaged) it is unclear if the alternate conformation that putatively triggers termination represents one conformation of a two-state system or a single point in a continuum of conformational states. Our data supports the later scenario for phi29 motor regulation since we observe a continuous decrease in velocity for individual complexes and large heterogeneity among an ensemble of complexes (23). Mutant studies also indicate that residue changes in the portal protein can affect the length of DNA packaged (40). Unlike phi29, P22 packages by a "headful" mechanism in which the motor must excise a unit-length genome from a concatenated DNA substrate (3, 4, 37). The motor is triggered to terminate packaging and cleave the DNA after an appropriate genome length has been packaged. It was proposed that the conformational change observed in the P22 portal may be the signal that triggers the termination (37). CryoEM and molecular dynamics studies of phage epsilon15 also revealed a well-resolved ring of averaged density located inside the narrow groove at the base of the portal (36, 41). In simulations this groove section often consists of variable arrangement of two or more independent DNA segments (23). Cryo-EM studies of phi29 virions also reveal a ring of DNA in contact with the portal, although it is touching the "top" of the portal rather than being wrapped around it (34). Although phi29 does not package by a headful mechanism, an analogous conformational change in its portal, induced by contact with the packaged DNA, could communicate the allosteric regulatory signal to the motor protein.

Distinction between the effects of load and allosteric regulation
While we have distinguished motor slowing due to internal force that directly loads the motor from that due to allosteric regulation, the latter could still be caused by conformational changes induced by the buildup of internal forces. As illustrated in the schematic diagram in Fig. 6a and b, the force per unit area (pressure) exerted by the packaged DNA on the inner surface of the prohead wall and portal ring is presumably similar or equal, on average, to that resisting translocation of DNA by the motor through the portal channel. However, our findings show that the onset of the slowing due to the allosteric mechanism occurs at a much lower filling than slowing due to force directly loading the motor. If the allosteric mechanism is indeed triggered by internal force it must be much more sensitive to the magnitude of the force (i.e., triggered by lower forces than are needed to significantly slow the motor via load). Specifically, as discussed below, analysis of motor slipping reveals that the allosteric effect begins to significantly slow the motor well before any changes in load-dependent behavior are detected (i.e. slipping). Although we could not detect any internal force below ~70% filling it is likely that a small internal force (e.g., <1 pN, below that our slipping analysis can detect) does build up, as expected theoretically. Such low force would not significantly slow the motor via load (Fig. 5a), but could be more than sufficient to trigger motor slowing via the allosteric mechanism.

Effect on motor-ATP interactions

Our finding that motor restart time following exchange from γS-ATP to ATP increases dramatically with prohead filling shows that the motor-ATP interaction is affected. In principle, either a decrease in ATP binding rate or an increase in ATP dissociation rate would slow the motor (12), but our measurements rule out the latter possibility, as follows. Fig. 2d shows that the restart time at 70% filling is ~30 seconds, but at this filling the motor velocity during packaging is ~50 bp/s, which implies that one ATP is being hydrolyzed every 0.04 s (since ~2 bp are packaged per ATP) (12). Thus, ATP binding, hydrolysis, DNA
translocation, and ADP and phosphate release must all happen very rapidly in 0.04 s. The 30 s restart time we measure must therefore be attributed to slow dissociation of γS-ATP. Our measurements thus imply that the γS-ATP dissociation rate decreases with increasing prohead filling. Since γS-ATP has been shown to mimic ATP in its binding kinetics (12), this implies that if ATP had the opportunity to dissociate (as opposed to being rapidly hydrolyzed) its rate of dissociation would decrease with increasing prohead filling. A decreased dissociation rate, however, would increase motor velocity, and therefore cannot explain the observed reduction with filling. Therefore, we attribute the reduction in motor velocity to a decrease in the ATP binding rate.

A decrease in ATP binding rate could occur due to either weakening of the motor-ATP interaction or to occlusion of the ATP binding pocket. Our evidence that the rate of ATP dissociation decreases with increasing filling suggests that it is due to occlusion. Such occlusion could be caused by a conformational change in the motor protein that affects the ATP binding pocket or conformational changes in the prohead, portal, and/or pRNA components that result in steric hindrance of the entry of ATP into the binding pocket.

**Contributions of load vs. allosteric regulation to motor slowing**

The magnitude of internal forces implied by our measurements of motor slipping (Fig. 4c) are significantly lower than those estimated previously under the assumption that internal force was the only factor slowing the motor (11, 13, 16). This discrepancy is explained by our present finding that prohead filling has an independent allosteric effect on motor function. Our measurements of motor slipping suggest that the internal force is quite low (<1 pN) until ~70% filling, after which it rises sharply. The ~50% decrease in motor velocity going from 0 to 70% filling is therefore almost entirely attributable to allosteric regulation.

In Fig. 7a, we compare the measured dependence of motor velocity vs. filling to that which would be expected due to the buildup of internal force loading the motor alone. The
change in velocity was inferred from relating the inferred internal force-filling relationship in
Fig. 4c to the measured force-velocity relationship in Fig. 5b. This comparison shows that
allosteric regulation begins slowing the motor significantly at ∼20% filling, whereas slowing
due to the buildup of internal force starts much later, at ∼70% filling. We also plot for
comparison the overall mean packaging rate, which includes the effect of motor pauses and
slips (Fig. 7a). At low filling (<50%) pauses and slips cause negligible percent change but
pauses steadily increase in importance at high filling (see also Fig. 3b inset).

Overall, the allosteric mechanism, which both slows the motor velocity and causes
pausing, has a much larger effect on packaging kinetics than internal force. Comparisons of
the ratio of the initial velocity \(V_{\text{max}}\) to the expected velocity if force alone slowed the motor,
and to the ratio of \(V_{\text{max}}\) to the measured velocity, are plotted in Fig. 7b. This shows that
internal force alone is predicted to have only a minor impact on the overall velocity change,
accounting for a ∼1.5-fold reduction from the initial value, whereas the actual reduction
reaches ∼8-fold near the end of packaging. Thus, the allosteric interaction ultimately slows the
motor velocity by an additional ∼5-fold. The reduction in overall packaging rate, which
includes the effect of pauses, reaches ∼20-fold near the end of packaging. Thus, allosterically-
induced pauses ultimately slow packaging by an additional ∼2.5-fold.

**Force resisting DNA confinement and comparisons with theories**

Our findings shed new light on discrepancies between our earlier higher estimates of
forces resisting packaging (internal force) and theoretical predictions (7-9, 13, 16, 42-44). Our
present measurements of slipping suggest very little buildup of force from 0 to 70% filling,
followed by a rise to ∼23 pN near the end of packaging. This trend is in closer accord with
theoretical predictions based on continuum mechanics models and measurements of forces
driving DNA ejection (45). Specifically, Tzil et al. calculate an initially low rise and
maximum force of ∼25 pN based on a model assuming an inverse-spool conformation of DNA
and interaction potential derived from measurements of DNA condensation by osmotic pressure (7). Caveats in this comparison are that their study modeled phage lambda, which has a different shape and genome length and slightly higher packing density, considered slightly higher ionic screening, and did not consider non-equilibrium effects (23, 46). Calculations by Purohit et al. using a similar model, but specifically considering the phi29 prohead size, shape, and packing density, also predict a similar shape of the force vs. filling curve, though they do not make absolute predictions of the forces independent of previously reported experimental estimates (8).

Packaging has also been modeled using coarse-grained molecular dynamics simulations. Forrey and Muthukumar calculated a maximum resistive force of ~40 pN (9), which is higher than we find here, though again did not specifically model the phi29 prohead dimensions. Another caveat in this comparison is that DNA packaging was simulated at an initial rate ~10^5× faster than the experimental rate due computational constraints, which could cause larger deviations from equilibrium and higher forces. Petrov and Harvey also conducted molecular dynamics simulations using a DNA interaction potential derived from available osmotic pressure data for similar ionic conditions. They calculated resisting forces rising to ~60 pN (44). Again however, the simulated packaging was much faster than the natural speed (1 nm steps every 10 ns, although the authors point out that this timescale does not translate to experimentally measured time due the use of a coarse grained model for DNA). Again, faster packaging could explain larger deviations from equilibrium and higher forces. Specifically, the finding of higher forces in these simulations may be due to an inability of the simulations to model the very slow DNA relaxation dynamics which we have shown to occur on >10 minute timescales (23).

**Regulation responds to changes in both length and conformation of packaged DNA**
We have shown that packaging is not a quasi-static process because at high filling the DNA forms non-equilibrium conformations that relax on a timescale longer than the packaging reaction (23). When packaging was stalled at ~75% filling for ~12 minutes (on average), to allow the DNA to relax, the average motor velocity increased by ~23% after restarting. Notably, this change occurred with no change in prohead filling, indicating that this effect must be caused solely by a change in the conformation of the packaged DNA. At 75% filling the internal force implied by our measurements of slipping is <2 pN. According to the measured force-velocity relationship (Fig. 5b), at 75% filling the complete relaxation of 2 pN of force would only cause a ~4% change in velocity, so the observed velocity increase following DNA relaxation cannot be attributed to the relaxation of internal force alone. Thus, this acceleration must mostly be attributed to modulation of motor activity via the allosteric regulatory mechanism.

We also found that the frequency and duration of pauses decreased dramatically after the DNA was allowed to relax (23). Since we have shown here that pausing is not significantly induced by load force, the change in pausing dynamics after DNA relaxation must therefore be attributed to changes in the packaged DNA conformation that alter the interaction(s) responsible for the allosteric effect. Remarkably, these findings on both motor velocity and pausing imply that the allosteric sensor we have described responds not only to the prohead filling, but also to the conformation of the packaged DNA.

Earlier studies by our lab (13) also showed that the decrease in velocity with filling is dependent on ionic conditions which can change the packaged DNA conformation (47, 48). In conditions where the DNA-DNA interaction is purely repulsive, higher screening (with Mg\(^{2+}\), cobalt hexamine\(^{3+}\), or spermidine\(^{3+}\)) results in a later onset of motor slowing, consistent with decreased internal pressure causing decreased allosteric regulation. In addition, we recently found that high levels of spermidine, sufficient to induce DNA condensation in solution
(changing the DNA-DNA interaction to partly attractive) causes significant slowing of the motor as well as more frequent and longer pauses even at low filling levels (<50%) (32). While this finding is contrary to theoretical studies that predicted reduced forces resisting DNA confinement, our interpretation is that attractive interactions exacerbate the formation of highly non-equilibrium DNA conformations that result in higher resistance forces and/or greater allosteric slowing (34).

**Relationship with motor stepping kinetics**

Previously it has been shown that packaging occurs in rapid bursts of four 2.5 bp steps separated by dwells (18). At low filling the duration of the bursts is independent of ATP concentration but increases with increasing applied force, whereas the duration of dwells depends on ATP concentration but not applied force. A study published very recently by S. Liu et al. reported that the duration of the dwells also increases with increasing prohead filling (26). The conclusions we reach here are consistent with these findings. First, the reported increase in the duration of dwells with increasing prohead filling causes a load-independent decrease the motor velocity, consistent with our findings. Second, our measurements of motor restart time following nucleotide exchange imply force independent changes in motor-ATP interactions and suggest that motor slowing is due to slowed ATP binding. Previous measurements showed that motor velocity vs. [ATP] follows Michaelis-Menten kinetics (12). Liu et al. extended these measurements to show that the maximum velocity ($V_{max}$), Michaelis 7constant ($K_M$), and their ratio ($V_{max}/K_M$) decrease with increasing filling, suggesting that filling slows ATP binding, consistent with our conclusion. Third, our measurements of slipping frequency vs. filling and vs. force suggest internal force remains low (<1 pN) until ~70% filling and then rises steeply to ~23 pN near the end of packaging. Through a different method, Liu et al. infer internal force by relating the dependence of the duration of bursts of translocation steps on filling to their dependence on applied force, yielding a force which rises
in a similar manner to a maximum value of ~20 pN (26). That these two different methods of determining internal force yield very similar values provides strong support for the validity of these results.

Conclusions

We have presented three different findings which clearly indicate that prohead filling has a strong and indirect effect on the function of the motor distinct from the effect of a direct load force. First, motor-ATP interactions are strongly perturbed by filling but not by load. Second, motor pausing increases sharply with increasing filling but not with load. Third, the maximum internal force resisting packaging inferred from measurements of motor slipping is several-fold lower than previous estimates based on motor velocity, which implies that much of the reduction in motor velocity is independent of load. The implication of these findings is that an allosteric signal acts to continuously reduce the packaging rate even prior to the buildup of significant internal force. This signal is propagated ~100Å from the interior of the prohead to the motor protein mounted on the exterior.

Remarkably, the allosteric sensor responds to not only the quantity of packaged DNA but also its conformation, an effect which we propose mitigates the formation of highly non-equilibrium DNA conformations which we have shown can slow and stall the motor and cause the DNA to slip out (23, 32). Specifically, when the DNA is given less time to relax towards equilibrium this mechanism slows the motor and induces pauses. Conversely, the motor speeds up and pauses less when the DNA is given more time to relax (23). Thus the motor speed appears to be tuned by this regulatory mechanism to achieve sustainably fast packaging rates dependent on filling level and DNA conformation.

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Chapter 8, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in Biophysical Journal. (N. Keller, Z. T. Berndsen, D. E. Smith) (2015). Continuous Allosteric Regulation of a Viral Packaging Motor by a Sensor that Detects the Density and Conformation of Packaged DNA.
Figure 8.1 Viral Components of Phi29. Components of the phi29 packaging complex. Arrangement of the packaging components based on superposition of the cryoEM structure of the portal-pRNA-motor complex (after Ref. (24)) onto that of the fully packaged virus (after Ref. (25)). The arrows schematically illustrate that both internal and applied forces exert loads on the motor at the site where the motor grips the DNA, opposite to the direction of translocation. Note: the dimensions of the prohead shell are 42×54 nm.
Figure 8.2 Nucleotide Exchange Experiments. (A) A schematic illustration of the experimental setup. A prohead-motor complex is attached to one microsphere and held in one optical trap (left) and a single DNA molecule is attached by one end to a second microsphere and its other end is packaged into the prohead. (B-C) Examples of nucleotide exchange experiment. The motor is stalled by addition of g-S-ATP and then ATP is reintroduced and the motor is observed to restart after a delay ("restart time"). (B) An example of a measurement at low filling (22% genome packaged) showing a short restart time of 5 s. (C) An example of a measurement at high filling (75%) showing a very long restart time of 110 s. (D). Mean time to restart following nucleotide exchange. Dependence of mean restart time following exchange from g-S-ATP to ATP on prohead filling (n=304 packaging events). Error bars indicate standard error in the mean.
Figure 8.3 Analysis of Motor Pausing. (A) Mean frequency of pauses (number detected per kbp packaged) vs. prohead filling (n=45). (B) Mean frequency of pauses vs. applied force (n=130). Inset shows the % time spent paused. All error bars indicate standard error in the mean.
Figure 8.4 Analysis of Motor Slipping. (A) Mean frequency of slips (number detected per kbp packaged) vs. prohead filling measured with a 5 pN force clamp (n=320 packaging events). (B) Mean frequency of slips vs. applied force measured at low filling (<20%) in both force clamp mode (where force is held constant at different preset values; circles, n=190) and fixed trap positions mode (where force is allowed to build as packaging proceeds; squares, n=130). The solid line is a linear fit to the data points. (C) Force resisting packaging vs. prohead filling inferred from plots a&b. All error bars indicate standard error in the mean.
Figure 8.5 Motor Velocity Measurements. (A) Mean velocity vs. prohead filling (n=45). (B) Mean velocity vs. applied force (n=74). All error bars indicate standard error in the mean.
Figure 8.6 Two Different Mechanisms of Regulation. (A) Forces acting directly on the section of DNA entering through the portal exert a direct load on the portion of the motor that grips and translocates the DNA (dashed circle). (B) DNA packaged inside the prohead interacts with the inner wall of the prohead and portion of the portal protein which protrudes into the interior. This interaction allosterically regulates the motor protein attached to the exterior portion of the portal, in a manner distinct from direct load. Images constructed as in Fig. 1.
Figure 8.7 Factors Influencing Motor Velocity. (A) Measured mean packaging rate (solid line) and measured mean motor velocity (rate not including pauses and slips, dotted line) vs. filling compared with the velocity that would be expected if the force plotted in Fig. 6b was the only factor slowing the motor (dashed line). (B) Fold-decrease from initial value in measured packaging rate (solid line), measured motor velocity (dotted line), and expected velocity due to force alone (dashed line) Inset shows zoomed in plot of the dashed line. All error bars indicate standard error in the mean.
References


Chapter 9

The Viral Packaging Motor of Bacteriophage Phi29 Exhibits High Energy Efficiency and Large Force Generation
Abstract

During the assembly of many dsDNA viruses, a powerful molecular motor is required to compact a DNA polymer into a prohead to near crystalline density, overcoming the forces that arise from electrostatic repulsion, bending energy, and entropy loss. While the theoretical maximum force the phi29 motor can generate if the energy released by ATP hydrolysis were all converted into mechanical work is ~140 pN, experiments have only been able to put a lower bound on this figure and, furthermore, previous estimates for forces larger than 100 pN based on velocity vs. filling measurement need revision in light of the more recent results described in Chapter 8. The largest directly applied force, at low filling, is 57 pN so this must be considered the present lower bound. Here we conduct single molecule packaging measurements with optical tweezers to investigate the force at which the motor of bacteriophage phi29 stalls. We accessed loads larger than 57 pico-Newton (pN) with greater efficiency using a force ramping technique. At low prohead filling levels, we find that as the force is ramped up we can reach a maximum force of ~63 pN with detectible DNA translocation before the tethered DNA enters the overstretch region and the signal becomes too noisy to compute the packaging rate. To access higher loads, we ramped up the force at high filling levels where the packaged DNA provides an additional load on the motor using our recently revised estimates for internal force. We observed ~1 s episodes of packaging with loads as a large as ~89 pN at high filling levels where the packaged DNA loads the motor by more than ~20 pN. This value is 56% higher than the previous lower bound of the stall force based on external applied forces alone, and is the highest force ever detected for a viral packaging motor. Our measurements show that up to 64% of the available chemical energy is converted into mechanical work making phi29 motor one of the most efficient DNA translocating motors ever measured. It should be noted that we are still working on a journal
paper describing these findings, so the conclusions and figures in this chapter are to be considered preliminary.

**Introduction**

DNA packaging is a required step in the infection cycle of many dsDNA viruses. The viral genome before packaging is a semi-flexible, negatively charged polymer with a persistence length of ~50 nm. The DNA is packaged into a pre-assembled prohead, which is ~140 times smaller than the genome, to near crystalline density (~0.5 g/mL) in ~5 min at room temperature (~23 C) [1,2]. Packaging is achieved by a molecular motor that converts the chemical energy released by ATP hydrolysis into mechanical work. In order to package the DNA, the motor must overcome the energetic penalties that arise from electrostatic self-repulsion, bending and entropy loss. In the virus studied here, bacteriophage phi29, the genomic length is 6.6 µm and the prohead’s dimensions are 54 nm x 42 nm. Phi29 is an excellent model system to investigate the physical and biochemical processes involved in DNA packaging because of its highly efficient and simple, in-vitro bulk packaging assay. This approach has been carried over to studies using optical tweezers allowing for direct manipulation of single prohead-motor complexes [1,3].

Previous measurements with optical tweezers of the phage packaging motors, lambda, T4, and phi29, have shown that they can all generate at least ~40 pN of force and package at rates between 100-2000 bp/s [3-5]. While the chemical states of each of the motors may differ during their ATP hydrolysis cycle, all motors exhibit a monotonic reduction in motor velocity with increased applied force indicating that, at saturating ATP levels, the force-generating translocation step is the rate limiting transition. Additionally, measurements with lambda and phi29 show that the rate is reduced with increasing filling because the packaged DNA presents resistance to the motor [3,4].
Measurements of viral DNA packaging with optical tweezers have led to many theories that either predict the energetics and dynamics of packaging or predict the mechano-chemical cycle of the molecular motor during the ATP hydrolysis reaction [6-8]. The response of the motor to force is critical for the development of these packaging theories. For example, two important relationships that theoretical studies use to test the validity of their models are the profiles of the internal force versus filling curve and the motor velocity versus force curve. However, agreement between theory and experiment is weak indicating that more experimental work is needed.

Cryo-electron microscopy measurements have revealed that the motor is composed of three multimeric rings that are sandwiched in a coaxial geometry where the DNA is transported through the center of each ring. Attached closest to the prohead at the site for DNA entry is a head-tail connector ring composed of gp10 proteins. The remaining structure of the motor below the connector, which is responsible for hydrolyzing ATP and generating forces, is composed of an oligomeric ring of identical copies of pRNA and a pentameric ring of gp16 proteins. Sequence homology of gp16 indicates that the phi29 motor is part of the ASCE superfamily of molecular motors [9].

We have shown through single molecule measurements that the observed deceleration of the motor (~ 60% reduction from initial value) during the first 70% of packaging is not due to internal force but rather due to an allosteric mechanism [10]. The allosteric mechanism is induced by prohead filling and slows the motor by slowing the rate at which an ATP molecule binds onto a motor sub unit. We also showed that this mechanism is responsible for motor pausing. After 75% filling, the reduction in packaging rate is due to both the internal force, which rises to only ~22 pN upon completion, and the allosteric mechanism. Force slows the motor by inducing motor slipping, which is where the motor loses grip with the DNA causing the DNA to rapidly eject out of the prohead [11].
Before the allosteric slowing of the motor was discovered, studies assumed that the slowing of the motor was entirely due to force [1,3,12]. For example, the 50% reduction in motor velocity at 60% filling was thought to be due to a 15 pN internal force because an applied 15 pN load reduces the velocity by 50% [1,3]. The discovery of the allosteric mechanism clearly shows that this reasoning is incorrect. The 50% reduction in velocity at 60% filling is not due to load at all but instead a reduced ATP binding rate. Thus the conclusions regarding internal force that were drawn before the mechanism was discovered need to be re-visited. One conclusion is an estimate for the stall force.

An estimate of the phi29 stall force can be obtained from reference 1, which assumed that the reduction in motor velocity from filling was entirely due to load. If one subtracts the load that was inferred from filling, then the current estimate to date for the stall force for phi29 is ~57 pN. No studies to date have investigated whether phi29 or other viral molecular motors are capable of generating forces larger than 57 pN. A stall force value of 57 pN has been used in many theoretical studies that either predict the forces driving viral DNA packaging and ejection or predict the mechano-chemical cycle of the motor during ATP hydrolysis [7,13-15]. If the motor were to generate more or less force, then perhaps some of these predictions need to be updated.

Here we present measurements of force generation of the phi29 motor using improved methods and an updated estimate of the internal force. We accessed loads larger than 57 pN using a force ramping technique. With ~18 pN of extra load provided by the packaged DNA at ~95% filling, we find that the motor can generate at least ~89 pN of force, which is 56% higher than the (revised) previous lower bound for the stall force (57 pN).

**Results and Discussion**

Packaging of single dsDNA molecules into single empty phi29 proheads was measured with a dual trap optical tweezers system as described in Chapter 1 and other recent
A DNA molecule is attached to one trapped microsphere and a prohead-motor complex is attached to a movable trapped microsphere (Fig 1a). Bringing the two microspheres close in the presence of ATP allows for the motor to begin packaging by reeling in the DNA. We measure the length of DNA translocated versus time and can adjust the force exerted on the prohead-motor complex by increasing or decreasing the trap separation. In this study, we exerted pulling forces that ranged between 5 and 68 pN.

Previous studies of motor force generation used a technique that is not efficient in collecting data at forces above 40 pN [1,3-6,12]. The distance between the microspheres is held fixed during packaging causing the force to continuously increase as packaging proceeds. While this technique is very efficient for collecting large sets of data at forces below 40 pN, it is inefficient for measuring forces larger than 40 pN because above 40 pN, the linkage between the prohead and anti body microspheres frequently breaks. We applied a new force measuring technique that allowed us to access large loads that have not yet been characterized. To access higher forces with greater efficiency, we initiated packaging with a low force (~5 pN) and monitored the force for 1.5s to confirm activity. If the complex was active (force rose), the force was ramped up to the high force set-point in less than ~0.5s. Packaging then proceeded until the tether was lost (Fig. 1B). With this new technique, we can readily access forces above 40 pN. We are able to confirm that a complex is initially active (before the force ramp) if we do not detect any packaging activity at high force. We recently applied this technique to phage T4 and we were able to apply loads as large as 60 pN and still measure packaging, which is ~10 pN higher than detected previously [18].

We used an optical tweezers system with increased spatial resolution. The system implemented differential detection, which, under our current experimental conditions, increases our signal to noise ratio by ~3-fold allowing for more accurate measurements. We compute the packaging rate by sliding a 1 second window to the length of DNA packaged vs.
time data. The motor velocity is the packaging rate with slips and pauses removed as described previously [18].

The motor at low filling (<10%) can generate more force than the previous estimate of the stall force (~57 pN). The average force reached for complexes that reached 60 pN (n=133 complexes) was ~63 pN (Figure 2, 3A). Some complexes packaged past 63 pN, which under our current experimental conditions is where the DNA undergoes a structural transition and begins to overstretch (Figure 2G). Unfortunately, we cannot accurately calculate the packaging rate in the overstretch region using a 1 second time window because the DNA packaged versus time data is very noisy [19]. However, the force does slowly increase, indicating that the motor can generate forces greater than 63 pN. The maximum force detected was 68 pN (Figure 2C).

We compared our packaging data to a previously established model that predicts the mechano-chemical cycle of the ATP hydrolysis reaction in phi29 (Figure 3B) [6]. It is able to predict the motor velocity as a function of applied force at known concentrations of ADP, phosphate ions, ATP, and temperature. Their model has not yet been tested at forces above 40 pN. We find good agreement between our data and their model at forces between 5 and 63 pN. Thus their model is able to accurately predict the kinetics of phi29 at forces above 40 pN.

We suspected that the motor could generate more than 69 pN of force because 84% (n=133 complexes) of complexes with an applied stretching force above 60 pN packaged a positive amount of DNA into their prohead before the linkage between the microsphere and tether broke. To access higher loads, we ramped up the force at filling levels larger than 85%, which is where the packaged DNA loads the motor by more than 10 pN [10,11]. To accomplish this, we used a 25.3 kb DNA construct that was prepared by PCR and had one of its ends labeled with biotin. We initiated packaging with this long DNA construct and kept the DNA tension below ~10 pN until 85% filling. Once we reached 85% filling, we increased the
pulling force (Figure 1C). We observed episodes of the motor generating forces that are larger than 69 pN at high filling levels. However, these episodes were short (1-10 seconds) (Figure 4) because the DNA tether slipped out of the prohead. We find that applying a high load at high filling levels causes most of the complexes to lose complete grip with the DNA. For example, only 15.5% of complexes with loads larger than 40 pN (n=541 trials, average time before slipping 4.5 ± 8.9 s) packaged a positive amount of DNA into their prohead. In over 50% of trials (n=268 complexes), the average amount of DNA that slipped out of the prohead as the force was being ramped or set was 1.8 kb or ~10% of the genome.

At high filling and high load, the motor is slowed by a combination of force and a reduced ATP binding rate [10,11]. The reduced ATP binding rate is due to the allosteric mechanism that was discussed earlier (Ch. 8). The mechanism is induced by filling and promotes motor pausing at filling levels larger than 75%. It has been shown that packaging is composed of burst and dwell phases [20]. During a dwell phase, all of the motor sub units release a single ADP molecule and bind a single ATP molecule. After each of the sub-units has an ATP molecule tightly bound, the motor enters the burst phase. In the burst phase, four sub-units will each hydrolyze an ATP molecule and translocate the DNA by 2.5 bp, which results in a net 10 bp step size. After ATP hydrolysis, a phosphate ion is released into solution and each sub unit is bound to an ADP molecule. The allosteric mechanism slows the motor by reducing the ATP binding rate and/or the ADP release rate during the dwell phase. The motor is ~45% likely to be in the dwell phase at filling levels between 85-100% [10]. The fact that we do not observe inhibition with a 40 pN load at low filling but do observe inhibition with a 40 pN load at high filling suggests that the motor most likely cannot tightly grip the DNA under high load when it is in the dwell phase. In other words, only ~85% of phi29 phages will not package to completion if the load during packaging reaches 40 pN.
The average force generated for complexes that generated at least 68 pN (total force which is the sum of the internal force and the applied stretching force) (n=30 trials, average time before slipping out = 12.4 ± 12.1 s) was 77 pN. The maximum packaging force was ~89 pN (Figure 5B), which is our estimate for the stall force. This estimate for the stall force is 56% higher than the previous estimate (57 pN). Since the energy released by ATP hydrolysis in our current packaging solution (0.5 mM ATP, 5 uM ADP, 5 uM Pi) is ~120 pN-nm and the motor step size is ~0.85 nm, the motor’s energy efficiency is at least ~63%.

In conclusion, we have shown that the phi29 motor can generate at least ~89 pN. The efficiency and force generation of phi29 are much higher than other translocating motors. Myosin, kinesin, and RNA polymerase generate only 3, 8, and 23 pN of force respectively and exhibit an energy efficiency of ~40% which is 30% smaller than our reported efficiency for phi29 [21].
Figure 9.1 Force Ramping Technique. (A) Viral DNA packaging was measured with a dual optical tweezers using a force ramping technique. (B) At low filling, the force was monitored for 1.5s to confirm activity (1) and if the complex was active, the force was ramped up by increasing the distance between the microspheres until the force reached the set-point (2). Packaging was then allowed to proceed (3) until the linkage between the antibody and DNA tether broke (4), whereupon the force dropped to zero. (C) For force ramps measurements at high filling, the DNA tension was kept below 10 pN until the prohead filling level reached 85% (1) whereupon the force was ramped up to the set-point (2). Packaging proceeded (3) until the linkage broke (4).
Figure 9.2 High Applied Load at Low Filling. (A-F) Three typical measurements of prohead-motor complexes packaging with an applied force greater than 57 pN at low filling (<10%). The top row (A-C) is applied force and directly below it is the same trace in units of DNA packaged (D-F). The maximum force reached for (A) was 67.5 pN, for (B) was 67.2 pN and for (C) was 69 pN. Note that when the DNA enters the overstretch region (>63.5 pN), the force signal becomes noisy and the slope of the force versus DNA extension (reduction in DNA compliance) lowers in magnitude (G).
Figure 9.3 Maximum Force Generated at Low Filling. (A) Histogram of the maximum force reached for the subset of complexes that generated more than 40 pN of force. The DNA tension was increased with less than 10% of the genome packaged. If the packaging rate during the last second of activity was greater than 10 bp/s, then the complex was marked as active. (B) Fit (red line) to a model that is described in Reference 6 (n=436 complexes).
Figure 9.4 High Applied Load at High Filling. Three typical traces of complexes packaging with a large applied force and at high filling. Following the same layout as in Fig. 2, the top row is total force, which is the sum of pulling force and internal load from the packaged DNA, and directly below it is the same trace in units of DNA packaged. The red arrows in D and E point to episodes of packaging.
Figure 9.5 Maximum Force Generated at High Filling. Histogram of the maximum force generated for complexes that generated more than 40 pN of force.
References


Chapter 10

The Viral Packaging Motors of
Bacteriophages Phi29 and T4

Package Condensed DNA
Abstract

Adding a sufficient amount of polyamines with a charge that is +3 or greater will cause DNA in solution to undergo a conformational transition from a worm-like chain to a condensed toroidal spool. Polyamines such as Spermidine$^{3+}$ and Spermine$^{4+}$, which can induce DNA condensation, are commonly found in both eukaryotic and prokaryotic cells at millimolar concentrations. Viral DNA packaging is a required process in the lytic cycle of many dsDNA viruses. A powerful molecular motor packages a charged polymer into a preformed capsid or prohead to near crystalline density. It remains unknown whether the motor can package condensed segments of DNA. Since the diameter of condensed DNA is at least 100 times larger than the diameter of the entry point for packaged DNA (portal channel ring), the motor must somehow unwind the toroid before it can push a DNA segment into its prohead. Using optical tweezers we show that single phi29 and T4 prohead-motor complexes package single condensed DNA molecules. In phi29, the rate is reduced by 13%, indicating that the load presented to the motor by condensed DNA is ~5 pN. In T4, the average packaging rate with zero load is ~890 bp/s, which is the first reported estimate for T4, and packaging with condensed DNA reduces this rate by 40%. The reduction in packaging rate in T4 is much larger than the reduction in phi29 because in T4 an increasing load causes frequent motor pausing and slipping. It should be noted that we are still working on a journal paper describing these findings, so the conclusions and figures in this chapter are to be considered preliminary.

Introduction

Viral DNA packaging is a required process in the lytic cycle of many dsDNA viruses. The viral genome is compacted into a prohead to near crystalline density by a powerful molecular motor. The motor, which is powered by ATP hydrolysis, must overcome the forces that arise from bending energy, entropy loss, and electrostatic repulsion. In bacteriophage phi29, which infects *B. subtilis*, the viral genome is 19.3 kbp and the dimensions of the
prohead are 54 x 42 nm [1]. In bacteriophage T4, which infects *E. coli*, the viral genome is 169 kbp [2]. Both phages package their genome in ~5 min at room temperature to similar final densities [3].

Optical tweezers studies have shown that both the T4 and phi29 motors can package DNA with applied tensions as large as 50 pN [4,5]. This force is at least 20 times larger than the forces measured with the skeletal muscle myosin making viral molecule motors one of the strongest ever measured [6]. In all of these studies, the force is either constant or slowly increasing during packaging.

Spermidine$^{3+}$ and Spermine$^{4+}$ are polyamines found inside prokaryotic and eukaryotic organisms at milli-molar concentrations and have been used as condensing agents in many DNA condensation studies [7-9]. In the presence of a critical amount of cations with charge +3 or greater, DNA in solution will undergo a conformational transition from a worm-like chain to a condensed toroidal spool. During DNA condensation, the DNA-DNA interaction potential changes from purely repulsive to partly attractive [10]. Bulk studies have shown that the diameter of the toroid ranges between 50-300 nm and that DNA toroids exhibit relaxation times that range between 10 min to several hours [11]. Analytical theories predict that the cross section of a DNA toroid exhibits hexagonal symmetry [12].

We can measure the elasticity of DNA by stretching single DNA molecules with optical tweezers. With repulsive interactions, the force monotonically rises with an increasing DNA extension and monotonically falls with a decreasing DNA extension. In other words, there is no hysteresis if the DNA tether is continuously stretched and relaxed. Much different behavior is observed with partly attractive DNA-DNA interactions during DNA stretching and relaxing experiments. When a condensed DNA molecule is being stretched, the force rapidly rises and falls. Its force versus DNA extension profile is similar to a saw-tooth wave (Figure 2) [13,14]. The saw-tooth behavior is believed to represent the unwinding of a DNA toroid.
Reference 24 measured the distance between adjacent force peaks to compute the diameter of a DNA toroid. They estimate an average diameter of 300 nm, which is consistent with some bulk electron-microscopic studies [12]. This finding also shows that a single DNA tether with both of its ends bound to trapped microspheres forms a toroid.

If the concentration of polyamines is high enough to induce DNA condensation during viral infection, then the external DNA is condensed and the motor must then be able to package condensed DNA. Comparing the difference in diameters between condensed DNA and the portal channels of phi29 and T4 suggests that in order for the motor to package condensed DNA, it must first de-condense the DNA toroid. The portal channel ring is the entry point for the DNA during packaging. The diameter of the portal channel ring for T4 (gp20) and phi29 (gp10) is ~0.3 nm, and the diameter of a DNA molecule in solution is ~0.25 nm. The minimum reported diameter of a condensed DNA toroid (~50 nm) is at least ~160 times larger than the portal channel [3,10]. Thus to de-condense the DNA, the motor must unwind the DNA toroid. One possible way is forcing the toroidal spool through the portal channel ring.

Some early studies have suggested that packaging with a concentration of polyamines sufficient to induce DNA condensation enhances packaging [15,16]. Reimer and Bloomfield found that the energy contributed by the DNA electrostatic repulsion is canceled by the contribution from polyamines. Schellman and Gosule state that, since the density of condensed DNA is similar to the density of DNA packaged inside of phage heads, then it is conceivable that DNA condensation by polyamines could be a significant driving force in packaging. However, they note that if the DNA was condensed inside the cell, then other cellular processes could not proceed.

Multiple studies that have investigated the effect of DNA condensation on the structure of packaged DNA [7, 9-10, 17, 21-23]. The packaged DNA inside T5 proheads forms
a structure that resembles a toroid if incubated in a solution of high Spermine$^{4+}$ [23]. Small ax-ray scattering measurements were done to measure the DNA-DNA spacings of partially (78%) and fully packaged lambda phages in the presence of high Spermine$^{4+}$ [17]. Molecular dynamical simulations of viral DNA packaging with partly attractive DNA-DNA interactions predicted reduced packaging forces and a final DNA structure that is a folded toroid [23]. However, for any of these studies to offer any conceivable biological implications, it must first be shown that viral packaging motors are actually capable of packaging externally condensed DNA.

**Results and Discussion**

The optical tweezer apparatus was assembled as described previously [17]. Phi29 motor protein (gp16) and proheads were prepared as described previously [18]. T4 motor protein (gp17) and T4 heads were prepared as described previously [4]. The DNA tether used for stretching measurements was prepared by PCR from lambda DNA. Its length is 25,339 bp and the opposite ends are labeled with biotin for tethering with streptavidin coated microspheres as described previously [18]. The DNA substrate that was used for packaging measurements is the same substrate as was used in the DNA stretching experiments, but with only one of its ends labeled with biotin. This substrate was also prepared by PCR.

Previous single molecule studies on DNA condensation have not yet determined the amount of Spermine$^{3+}$ required to condense DNA in the presence of millimolar concentrations of Sodium$^+$ and Magnesium$^{2+}$ ions. These studies only measured the amount of Spermine$^{3+}$ or Spermine$^{4+}$ required to condense DNA in a low ionic strength Sodium buffer (~10 mM NaCl, 10 mM TrisHCl pH 7.5). The phi29 packaging buffer contains 50 mM Sodium$^+$ and 5 mM Magnesium$^{2+}$ ions and so before we could begin our packaging measurements, we first needed to characterize DNA condensation as mediated by Spermine$^{4+}$ in the presence of the phi29 packaging buffer.
We first captured two streptavidin-coated microspheres with the optical tweezers and then briefly exposed them to a capillary tube in which a solution containing DNA stretching constructs were slowly flowing out of. During this exposure, the biotinylated ends of a DNA molecule will bind to the surface of the captured streptavidin coated microspheres. The trapped microspheres are then moved away from the capillary tube to the center of the flow chamber to conduct DNA stretching measurements. A gentle flow of solution buffer is applied during stretching measurements. We first stretched and relaxed the DNA molecule in 1x PBS solution (10 mM Na$_2$HPO$_4^{3-}$, 137 mM NaCl, and 2.7 mM KCl, pH 7.5), where the DNA-DNA interaction is purely repulsive, to insure that the DNA molecule behaved as a worm-like chain (Figure 1B). According to the worm-like chain model, the force monotonically increases with increasing DNA extension and decreases with decreasing DNA extension [14]. We then switched the ionic solution buffer to a solution containing 0.5x TMS solution (50 mM NaCl, 5 mM MgCl$_2$, and 25 mM TrisHCl, pH 7.5) plus 0.5 mM Spermine$_{4+}$, which induces DNA condensation. The minimal concentration of Spermine$_{4+}$ required for condensation in 0.5x TMS solution is ~0.1 mM. The DNA was stretched and relaxed in the high Spermine$_{4+}$ solution with a stretching rate of ~50 nm/s (Figure 1B). We found that the force needed to decondense condensed DNA ranges between 0.5 and 10 pN and unlike the repulsive regime in which the relaxation and stretching curves follow the same path, the stretching and relaxing curves follow different paths. These results are consistent with previous single molecule studies and indicate that the DNA condensate has not yet reached its equilibrium conformation [14]. The fact that the condensation force varies in magnitude implies that, in the context of packaging, the loads presented to the motor by condensed DNA should also vary in magnitude. We expect the motor to easily generate enough force to unwind condensed DNA, but unwinding a toroid by applying a stretching force is different than unwinding a toroid by pulling it through the portal.
channel. The motor cannot apply an end-to-end stretching force to de-condense the DNA. Instead, it must pull the condensed DNA through the portal channel, which has a much smaller average diameter than the smallest measured diameter of condensed DNA.

To test whether T4 and phi29 prohead-motor complexes could package condensed DNA, we performed a rapid solution exchange technique (Figure 2). Packaging was first initiated in 0.5x TMS and 0.5 mM ATP with a 5 pN applied load and then rapidly moved to a capillary tube that was flowing 0.5x TMS plus 10 mM Spermine$^{4+}$ and 0.5 mM ATP. In the high Spermine$^{4+}$ solution, the distance between the trapped microspheres was reduced to less than 2000 nm to induce DNA condensation of the external, unpackaged DNA. The external DNA forms a condensed DNA structure at this relative microsphere distance because our DNA stretching measurements in high Spermine$^{4+}$ show that the DNA becomes condensed at larger microsphere distances (Figure 1B). We confirmed that this protocol does not cause unpackaged, condensed DNA to stick to the trapped microspheres within the timescale of the experiment (Figure 2C). After incubating the complex in the high Spermine$^{4+}$ solution for ~15-20 seconds to give the motor time to package condensed DNA, we rapidly moved the complex to a region of the flowchamber that contained only the standard packaging solution (0.5x TMS and 0.5 mM ATP). The DNA de-condenses in less than a second once it is in a solution containing only 0.5x TMS. The DNA tension was then increased to 5 pN. The change in DNA extension divided by the high Spermine$^{4+}$ incubation time equals the average packaging rate.

For phi29, we conducted these measurements across all filling levels to generate a packaging rate versus filling curve (Figure 3). For example, to estimate the reduction in packaging rate due to condensed DNA at 60% filling, we tracked packaging to 60% filling with a 5 pN load in the standard packaging solution, and then reduced the microsphere distance and moved the complex to the high Spermine$^{4+}$ solution for ~15-20 s.
We found that both motors package condensed DNA. In phi29, the packaging rate with less than 10% of the genome packaged is reduced by $13\% \pm 1.7\%$ ($n = 75$ complexes), which implies that the condensed DNA presents a ~5 pN effective load to the motor [5]. The motor packages condensed DNA across all filling levels showing that the sum of the external load, which is due to the condensed DNA, and the internal load, which is due to packaged DNA, does not exceed the motor’s maximum force. Similar to our previous single molecule measurements with high Spermidine$^{3+}$, the packaging rate is roughly constant between 0-25% filling (Figure 3) [5]. This packaging rate plateau indicates that the resistance presented to the motor by the packaged DNA is nearly zero in this filling window. In contrast, the rate begins to drop at 5% filling in the standard packaging solution (0.5x TMS), where the DNA-DNA interaction is purely repulsive [5,17]. A packaging rate plateau was also observed in our measurements with high Spermidine$^{3+}$, low Spermdine$^{3+}$, and low Cobalt Hexamine$^{3+}$ (a low polyamine concentration represents a condition where the DNA is not condensed and a high ionic polyamine concentration represents a condition where the DNA is condensed) [5,19]. We argued that the presence of the packaging rate plateau indicates that the free energy of the packaged DNA is reduced or in other words, the packaged DNA adopts a closer to equilibrium conformation. In addition, we have also recently shown that introducing a high Spermine$^{4+}$ solution to complexes at filling levels between ~20% and ~65% immediately (~1s) increases the motor velocity [20]. This suggests that the Spermine$^{4+}$ ions rapidly diffuse into the capsid and favorably change the conformation of the packaged DNA. A favorable conformational change means that the confined DNA has a lower free energy and that the forces resisting confinement are lower. A separate study on phage lambda also found evidence for the confined DNA undergoing a favorable conformational change after exposure to a high Spermine$^{4+}$ solution. Using small X-ray angle scattering, they found a reduction in the DNA-DNA spacing
at filling levels up to 78% [21]. Thus, we believe that the presence of the packaging rate plateau indicates that the packaged DNA has undergone a favorable conformational change.

In phage T4, the packaging rate without applied load is $890 \pm 25.9$ bp/s ($n = 67$ complexes), which is the first reported estimate of the packaging rate with zero load for T4 (Figure 4). This value is 13% higher than the rate with a 5 pN applied load ($785 \text{ bp/s} \pm 24.6$ bp/s, ($n = 63$ complexes)), and similar to the percent increase measured in phi29 (8%) [17]. Packaging condensed DNA reduces the T4 motor velocity by 42% ($515 \pm 22$ bp/s), which is ~3-fold larger than the reduction measured in phi29 and indicates that the condensation force is ~9 pN. The increased reduction in packaging rate with T4 is most likely due to the fact that in T4 the heterogeneity in packaging rate increases with increasing applied load [4,22,23]. The motor frequently pauses and slips at forces above 5 pN. For example, our recent T4 measurements, have shown that with a 15 pN applied load, the motor spends 40% of its time in the paused or slip state [4]. Consequently, the packaging rate, which includes pauses and slips, is 43% smaller than the motor velocity. Phi29 does not exhibit this unusual behavior and as a result the motor velocity is equal to the packaging rate at all applied loads [5]. It has been suggested that the heterogeneity at large forces could be reduced by including the small terminase subunit (gp16) in the in-vitro packaging assay [23]. It currently is not included because it reduces the in-vitro packaging efficiency to below a level where the single molecule assay is feasible [24]. Therefore, it is best to use the reduction in velocity that is measured with phi29 to estimate the load presented to the motor by condensed DNA.

In conclusion, we have shown that both phi29 and T4 package condensed DNA segments. Our results indicate that the effect of external DNA condensation on packaging is not severe, and published theoretical studies that neglected this effect were making a reasonable approximation.
Figure 10.1 Stretching Single Condensed DNA Molecules. (A) A single DNA molecule is attached to two trapped streptavidin coated microspheres. To stretch the DNA, the distance between the microspheres is increased. The diameter of each microsphere is ~2200 nm. (B) Stretching and relaxing a condensed DNA tether with optical tweezers. Relaxation and stretch curves in high Spermine$^{4+}$ are in green and red, respectively. The time between stretches and relaxations was ~0.1 s. The initial stretch and relaxation curves were carried out in 1xPBS solution and they are in black and blue, respectively.
Figure 10.2 Protocol for Testing Packaging with Condensed DNA. Overview of the protocol that tests whether single prohead-motor complexes are able to package condensed DNA. (A-B) Packaging is initiated in 0.5x TMS solution with a 5 pN applied load (1) and then moved to the high Spermine$^{4+}$ solution (2). In the high Spermine$^{4+}$ solution, the DNA tether is relaxed to promote condensation of the external, unpackaged DNA (3). The DNA remains relaxed for ~15-20s to allow for packaging. The complex is then rapidly moved back to the 0.5xTMS solution (4) and the force is increased back to 5 pN (5). In (A), the grey section represents the high Spermine$^{4+}$ solution and the white section represents the 0.5x TMS solution. (C) A condensed DNA tether that is relaxed in High Spermine$^{4+}$ does not stick to the microspheres. The packaging trace in (B) was carried out with phi29 prohead-motor complexes.
Figure 10.3 Packaging Rate vs. Filling with Condensed DNA. Packaging rate of phi29 versus prohead filling with zero applied load and in high Spermine$^{4+}$. 
Figure 10.4 Phage T4 Packaging Condensed DNA. The packaging rate of single T4 prohead-motor complexes was measured with a zero load in 0.5x TMS (A) and in High Spermine\(^{4+}\) (B). In (B), the oscillation in the signal at ~2s was introduced by the user. To measure packaging in condensing conditions, the user must physically move the packaging complex to a region of the flowchamber that contains high Spermine\(^{4+}\). See Figure 2A for a schematic of the experiment.
References


Chapter 11

Reducing Torsional Rigidity of the DNA Substrate during Motor Driven Packaging in Bacteriophage Phi29
Abstract

In viral DNA packaging, the motor packages a charged polymer into a pre-formed capsid or prohead to near crystalline density in only ~5 minutes, overcoming the forces that resist tight polymer confinement, which are due to entropy loss, bending energy, and electrostatic repulsion. If significant twisting of the DNA occurs, it may also involve a torsional twisting energy. Many theories assume zero torsional rigidity as this is easier to model and it is assumed that little twisting would occur during packaging, or if twisting did occur, the DNA would relax rapidly to a near zero torsional energy state. However, some models have considered torsional rigidity and the possibility that the motor could twist the DNA as it is translocated and the possibility that torsional relaxation may not freely occur, and do predict some effects on packaging. No experimental study to date has specifically probed for the effect of torsional rigidity on packaging. We prepared a nicked DNA packaging substrate that had approximately one nick per every 1.7 persistence lengths. The site of the single stranded nick can be interpreted as a freely flexible joint so packaging with nicked DNA reduces the energy associated with DNA bending and DNA twist. We then used optical tweezers to measure single phi29 proheads package this single nicked DNA substrate. If torsional rigidity plays an important role, then packaging is either significantly enhanced or inhibited. We find that the motor velocity vs. filling profile is not significantly altered between ~0% and 95% filling. Roughly ~40% of complexes stall before reaching 95% filling. These results suggest that a reduction in torsional rigidity and/or bending rigidity promotes the formation of farther-from-equilibrium conformations that present a larger resistance and stall the motor. In addition, we find that a single stranded nick causes the motor to enter a long reversible pause state which ranges between 0.2-300 seconds. In the context of viral infectivity, our results suggest that a nicked DNA molecule reduces the overall infectivity rate because both motor stalling and the time required to package to completion are increased. It
should be noted that we are still working on a journal paper describing these findings, so the conclusions and figures in this chapter are to be considered preliminary.

**Introduction**

Viral DNA packaging is a fundamental process in the lytic cycle of many dsDNA viruses. The genome is packaged into a pre-formed prohead to crystalline density by a molecular motor that converts the energy released by ATP hydrolysis into mechanical work. The motor must overcome the forces that arise from tight polymer confinement which are due to entropy loss, bending rigidity, and electrostatic repulsion. In the virus studied here, phage phi29, the genomic length is 6.6 µm and length of the prohead is ~50 nm [1]. Phage phi29 is an excellent model system to study the dynamics of tight polymer confinement because its in-vitro packaging assay is highly efficient and robust, and the heterogeneity in packaging dynamics among different individual complexes is very small.

According to almost all analytical models and molecular dynamics simulations, the bending energy during the early stages of packaging is the largest contributor to the internal force [2-6]. However at high prohead filling levels, these models find that the contribution from the bending energy is negligible compared to the other forces (due to either entropy or electrostatic repulsion) resisting DNA confinement.

DNA inside cells is generally in a torsionally rigid state and through the action of enzymes become torsionally constrained or relaxed. DNA supercoiling occurs when enzymes such as DNA gyrase increase the helical pitch causing DNA to contort to a new shape. Torsion of the DNA can be relieved by topoisomerase, which nicks the DNA at certain sites such that the DNA can be replicated or transcribed properly [7]. A single phosphodiester bond of the DNA molecule is broken at the location of the nick. The site of a nick acts approximately as a freely flexible joint and therefore nicking a DNA molecule reduces the energies associated with bending and DNA twist (torsion).
Most theorists assume that the torsional rigidity of a DNA molecule doesn’t play a large role in dictating the conformation of the packaged DNA and thus do not include it in their models [3,4,8]. They assume that either the motor does not rotate the DNA as it is translocating into the capsid or that the relaxation time of a torsionally constrained DNA molecule is within the timescale of packaging [4].

The role of torsional rigidity in packaging has only been investigated through modeling by two groups [9,10]. These two studies only considered whether increasing the DNA twist affects the dynamics and did not consider the case with a reduced DNA twist. There is not yet a consensus of whether reducing the torsional rigidity affects the final DNA conformation. No structural studies address this issue either. One study used molecular dynamical simulations and found that the forces resisting DNA confinement from a DNA substrate that is torsionally constrained are similar to the forces resisting confinement from a DNA substrate that is torsionally relaxed [9]. They conclude that since the DNA torsionally relaxes on a timescale similar to packaging, the torsional rigidity of the DNA does not alter the packaging energetics and DNA conformation.

Two bulk experimental studies have investigated if packaging efficiency is altered with nicked DNA. As stated above, nicking a single strand of the DNA substrate reduces both the torsional rigidity and bending rigidity because the site of a single stranded nick can be interpreted as a freely flexible joint. One study mixed nicked T5 DNA with pre-assembled T5 proheads and measured no reduction in packaging efficiency [10]. To nick the DNA, they incubated it with DNase I. Another study also used this procedure with phi29 and also measured no reduction in packaging efficiency [11]. However, since DNase I nicks DNA molecules at random sites, it is likely that the number of nicks in each DNA molecule varied. In other words, the distribution in torsional and bending rigidity of the packaged DNA is most likely very large. While their results suggest that a reduction in torsional rigidity does not alter
packaging, it is not conclusive and more experimental work is needed. More specifically, it remains unknown what affect a nicked DNA substrate has on the single molecule dynamics of viral DNA packaging (i.e. how motor velocity versus prohead filling, motor pausing, and motor slipping are affected).

We used optical tweezers to measure single nicked DNA molecules being packaging into phi29 single proheads. Phi29 proheads and gp16 motor protein were prepared as described previously [11]. The optical tweezer system was assembled as described previously [12]. The data were collected using a feedback-controlled force-clamp program that maintains a constant tension during packaging [13]. The non-nicked packaging substrate is a biotin end labeled, 25,339 bp DNA molecule that was prepared by PCR from lambda DNA as described previously [13]. To produce a DNA substrate with single stranded nicks, we digested the DNA with four nicking restriction endonucleases (all purchased from NEB Labs, Inc). This digestion theoretically nicks the DNA at 87 sites. The distance between adjacent nicks (ignoring bottom and top strand) is approximately 1.7 persistence lengths (85 nm). We used alkaline gel electrophoresis to confirm that our 25.3 kb packaging substrate was nicked. The number of nicks packaged versus filling is plotted in Figure 1. The fully packaged substrate, which is a 19.3 kb fraction of the 25.3 kb substrate DNA, has 68 nicks.

We also tested the efficiency of each nicking endonuclease by preparing a duplex oligo (100 bp) that had a single nick site on both strands (purchased from IDT, Inc.). The nick sites were located directly opposite each other. Digesting this duplex oligo with any of the four nicking endonucleases results in both sites being nicked, which causes the duplex oligos (100 bp) to break apart into two smaller DNA fragments (~50 bp). After incubating each enzyme with the duplex oligos, we quantified how much DNA was nicked by applying standard gel electrophoresis. We found that at least 90% of the duplex oligos were nicked by each enzyme,
suggesting that the majority of enzyme recognition sites in our DNA construct for packaging are nicked.

2. Results

At filling levels less than 70%, we found that the motor enters long pause states that range between 0.2-300 seconds (Figure 2). The motor typically pauses at filling levels larger than 70% but these high filling pauses are due to the packaged DNA and not the external, unpackaged DNA [14]. Pausing is almost never detected with a non-nicked DNA substrate at filling levels less than 70% [13]. Thus the low filling pausing with the nicked DNA substrate is due to an interaction between the motor and the single stranded nicks. The motor exits these pause states (or continues packaging) with an efficiency of 89.8 ± 1.3 % (n=579 pauses detected).

A similar affect was also observed in Reference 15 where low filling pauses were observed with packaging substrates that had single strand gaps. Through their high-resolution optical tweezer measurements, they showed that the motor is unable to grip single-stranded DNA. When the motor encounters a single strand site, it loses grip with the DNA causing the DNA to eject rapidly out of the capsid. As the DNA is ejecting out of the capsid, the motor regrips the DNA only when it encounters a double-stranded section. The amount of DNA slips out during this process is only ~10 bp. We would classify this slip-re-catch-package event as a pause because our resolution is only ~30 bp. The similarity in behavior we observe suggests that the nick-induced pauses that we observe are slip-recatch-package events.

The motor velocity, which we compute after removing pauses larger than 0.2 s, is slightly reduced by ~5% across all filling levels (Figure 3A). This reduction in velocity may be due to our inability to remove nick-induced pauses that are smaller than our bandwidth (0.2s). There is no change in the profile of the normalized velocity versus filling curve between nick and non-nicked DNA across all filling levels (Figure 3B).
However, we find that ~40% of complexes that packaged the nicked DNA substrate stall before 95% filling. In contrast, 17% of complexes that packaged with non-nicked DNA stall before 95% filling. This inhibitory affect is most likely not observed in our normalized velocity versus filling curve because the motor velocity in this region (15-20 bp/s) is only slightly above our resolution (10 bp/s).

The internal packaging force rises from ~10 pN at 90% to ~22 pN at 100% [14]. We considered if a combination of pulling force and nicked DNA weakens the motor’s grip with the DNA to a point where the motor stalls. To rule out this possibility, we measured packaging with our nicked DNA substrate and with an applied 30 pN load (Figure 4). The motor does not stall under these conditions, which clearly indicates that stalling is due to an effect of the packaged DNA conformation on the motor.

In conclusion, we have shown that a reduction in energy associated with DNA twist and a reduction in bending energy inhibits packaging only at filling levels above ~95%. Below ~95% filling, the conformation is not affected because there is no reduction in the deceleration of normalized motor velocity with increasing prohead filling. Above ~95% filling, we find that 60% of complexes continue to package. Our interpretation is that this inhibition is due to the packaged DNA forming a non-equilibrium structure that presents a resistance force that exceeds the motor’s maximum force.

We have recently shown in studies with a net attractive DNA-DNA interaction condition that the history of the packaged DNA affects the subsequent packaging dynamics (history dependent dynamics) [15]. This concept can be applied here to explain our results as well. The reduced bending energy with a nicked DNA substrate could allow the DNA at early filling levels to explore more phase space and become “locked” in a trajectory that leads to a non-equilibrium conformation. For the fraction of complexes that stall, the trajectory of the DNA leads to a very unfavorable, non-equilibrium DNA conformation that presents a very
high resistance force to the motor that the motor cannot overcome. However, for the complexes that do not stall, the trajectory of the DNA is similar to the trajectory followed with non-nicked DNA. Along this path, the resistance forces are smaller than the maximum force of the motor, which allows packaging to complete.

Our results also suggest that viral infectivity would be significantly lowered with a nicked genome because the amount of time needed for packaging to complete increases while the overall efficiency of packaging decreases (higher stalling rate) (Fig. 6).
Figure 11.1 Number of Nicks packaged vs. Filling.
Figure 11.2 Phi29 Complexes Packaging Nicked DNA. Example traces of single phi29 proheads packaging single DNA molecules that were nicked. The applied load during packaging was 5 pN. The long pauses are due to the motor not being able to grip the DNA at a nick site.
Figure 11.3 Velocity versus Filling of Nicked and Non-nicked DNA. Nicked DNA is the blue line and non-nicked DNA is the black line. In (A) the velocity is in units of basepairs per second and in (B) the velocity is normalized (normalized velocity = velocity divided by the maximum velocity (v/vmax)).
Figure 11.4 Phi29 Complexes Packaging Nicked DNA with High Force. Example traces of single phi29 proheads packaging the nicked DNA substrate with an applied 30 pN load, which is larger than the approximate load that the packaged DNA presents to the motor near 100% filling (~22 pN). The motor does not stall under these conditions indicating that the stalling observed at filling levels larger than 90% is due to the conformation of the packaged DNA.
Figure 11.5 Time vs. Length of DNA Packaged. Time to package versus DNA packaged for nicked (blue) and non-nicked DNA (black). Packaging with nicks causes motor pausing and thus lengthens the packaging time. Longer packaging times in combination with the finding that only 60% package past 95% filling suggests that nicked DNA substrates result in lower viral infectivity.
References


Chapter 12

Effect of Temperature on Viral DNA Packaging in Bacteriophage Phi29
Abstract

Viral DNA packaging is a fundamental process in the lytic cycle of many dsDNA viruses such as adenovirus and herpesvirus. The viral genome is compacted into a pre-formed prohead to crystalline density by a molecular motor. The motor is powered by ATP hydrolysis and works against the forces that resist tight polymer confinement which are due to bending energy, entropy loss, and electrostatic repulsion. Temperature is an extremely important parameter to investigate in biological systems because it can affect the conformation and dynamics of macromolecules such as DNA and proteins. Temperature also regulates the rate of chemical reactions that enzymes perform. While it has been shown that various viral hosts live at temperatures ranging between ~0 °C to 50 °C, the dynamics of DNA packaging by ATP-driven viral motors has not been studied in detail. Both the enzymatic activity of the motor and the dynamics of the DNA are likely affected. A recent study found evidence that DNA packed in phage lambda undergoes a solid-to-fluid-like transition with increasing temperature at ~37 °C that was speculated to facilitate ejection of the viral DNA by increasing its mobility in the densely packed state. Clearly this “solid” state, if it exists below 37 °C, does not prevent DNA packaging because packaging in phi29, lambda, and T4 does occur fine at lower temperatures. However, if a transition to a more fluid state occurred in phi29 it might make packaging easier at elevated temperatures by reducing the forces resisting DNA confinement. Our previous measurements only investigated packaging at room temperature (~22 °C). Here we conduct single molecule packaging measurements with phage phi29 at temperatures above and below room temperature. We find that the packaging rate increases 1.82 fold with a 14 degree temperature increase and decreases by 62% with a 14 degree temperature decrease. We show that the motor velocity and temperature are related through an Arrhenius-like dependence, which has also been shown in many other motors such as myosin and kinesin [1]. We extract a mean kinetic transition distance of 0.15 nm which is consistent
with previous studies on phi29 [2]. The reduction in normalized velocity with increasing prohead filling increases with increasing temperature. There is no increase in motor stalling with the imposed temperature changes. Our results suggest that increasing or decreasing the temperature drives the system slightly farther into or further out of equilibrium but not to a point that prevents packaging. It should be noted that we are still working on a journal paper describing these findings, so the conclusions and figures in this chapter are to be considered preliminary.

**Introduction**

Viral DNA packaging is a critical step in the lytic cycle of many dsDNA viruses. A charged DNA polymer is compacted into a pre-formed prohead to near crystalline density by a molecular motor. The motor, which is powered by ATP hydrolysis, works against the forces that resist tight polymer confinement which are due to bending energy, entropy loss, and electrostatic repulsion. In the virus studied here, phage phi29, the length of the genome is 6.6 \( \mu \text{m} \) and the dimensions of the prohead are 42 x 54 nm. The phi29 prohead-motor complex packages its viral genome in \(~5\) minutes at \(~20 \ \degree\text{C}\) [3,4].

Optical tweezers measurements have shown that the packaging rate of phi29 monotonically decreases with increasing prohead filling in part because the packaged DNA presents a resistance to the motor that increases with increasing DNA density [5,6]. Between 0-75% filling, the internal force is less than \(~5\) pN and the reduction in packaging rate is entirely due to an allosteric interaction between the packaged DNA and motor [7,8]. Through the allosteric interaction, the ATP binding rate of the motor is monotonically reduced with increasing DNA density. This has been shown through high resolution optical tweezer measurements conducted by the Bustamante lab at UC Berkeley [8]. The DNA translocation cycle consists of a burst phase and dwell phase. During the dwell phase, each motor sub unit releases an ADP molecule and then binds a single ATP molecule. During the burst phase, four
motor sub units each hydrolyze an ATP molecule and this results in a net 10 bp step-size (step-size of each sub-unit is 2.5 bp). The allosteric mechanism slows the motor by reducing the ATP binding rate and force slows the motor by lengthening the burst phase. Our findings suggest that the allosteric mechanism functions to regulate the DNA conformation by lowering the motor speed prior to the buildup of significant internal force [7]. It is assumed that if the rate of DNA entering the prohead is reduced, the packaged DNA has more time to relax into an equilibrium or near-equilibrium conformation. A conformation that is closer to equilibrium results in lower internal forces and larger motor speeds. Above 75% filling, the allosteric mechanism induces motor pausing, which ranges between ~1 s to ~10 s. By lowering motor speed, local regions and the global conformation of the packaged DNA are allowed to relax. Above 75% filling, the packaged DNA begins to load the motor. Upon completion of packaging, the internal force reaches a final value of ~22 pN [8].

Temperature regulates the rate of enzymatic activity and has recently been reported to induce conformational transitions in densely packed DNA [9,10]. In optical tweezers studies, temperature is rarely varied because the efficiency of enzymatic activity often significantly drops at sub-optimal temperatures, making experiments difficult, and/or it is difficult to control the temperature of a sample in an optical tweezers instrument. Bacterial host cells have been found to live at temperatures ranging between ~0 °C and 50 °C [11,12]. How the dynamics of viral DNA packaging are affected with altered temperatures remains an open question. In this present work, which uses optical tweezers to measure the single molecule packaging dynamics, we were able to measure packaging at temperatures between 10 °C to 38 °C.

Theoretical analyses of viral DNA packaging using continuum mechanical models have not explicitly discussed significant temperature dependent effects in the packaging dynamics in our temperature range (10 °C to 38 °C) [13,14]. In such models the behavior is
assumed to be independent of packaging rate and the energy of the packaged DNA scales linearly with absolute Kelvin temperature through the bending energy term [13-15]. Increasing or decreasing the temperature by 14 degrees would change the total internal force by less than ~5% within such a model. The Brownian motion of the DNA polymer is ignored in these analytical models.

DNA though, like other microscopic particles, is subject to Brownian motion. Concentrated polymers in an entangled solution will undergo reptation. If this mechanism applies to all packaged DNA molecules, which has been suggested in some studies, it would not predict a significant temperature dependence [16,17]. According to reptation theory, the longest relaxation time scale is the tube disengagement time, which is the time required for a polymer to completely diffuse out of its initial tube by reptation [18]. The tube disengagement time scales linearly with temperature, where temperature is in units of absolute Kelvin. A 14 degree increase/decrease in temperature would increase/decrease the disengagement time by only ~5%. The disengagement time is a lower bound estimate for the relaxation time because both ends of a polymer are free during reptation whereas during packaging one end is fixed while the other end is free.

On the other hand, it has been suggested that the DNA may form liquid crystalline states or disordered “glassy” states, and it is possible to imagine that temperature changes could induce sharp phase transitions in such systems [19]. Furthermore, recent experimental studies provide evidence that DNA packaged in phage lambda, at similar density as achieved in phi29, undergoes a solid-to-fluid-like transition as the temperature increases to ~37°C [9,10].

An increase in packaging rate due to an increase in temperature could cause the packaged DNA to be driven further out of equilibrium. Since we have already shown that the packaged DNA is out of equilibrium at room temperature, increasing the rate at which DNA
enters the prohead could drive the system farther from equilibrium because the packaged DNA has less time to relax. DNA conformations with increased free energy present higher resistance forces to the motor and result in reduced packaging rates. Allosteric down-regulation of motor velocity also appears to act in response to farther-from-equilibrium conformations since allowing for relaxation increases motor velocity [20]. If the resistance forces exceed the motor’s maximum force, then the packaged DNA will eject out of the prohead. Thus, increasing the temperature could inhibit packaging. Conversely, decreasing the temperature, which results in a lower packaging rate, could enhance packaging because the packaged DNA now has more time to relax.

Recent measurements on phage lambda have shown that, at 37 °C, the mobility of the packaged DNA increases because the electrostatic repulsion between adjacent DNA strands is reduced [9]. A combination of both single molecule and bulk methods were used to show this affect. Small angle x-ray scattering and calorimetric measurements revealed that the packaged DNA at 37 °C is much more disordered and that the intra-strand repulsion is reduced suggesting increased mobility of the packaged DNA. Packaged lambda capsids were also indented by using atomic force microscopy at temperatures that ranged between 22 °C to 37 °C. At 37 °C, the capsids with packaged DNA are as flexible as empty capsids, which suggest that the DNA is in a more fluid-like state. Above this transition temperature, the ejection rate is also significantly accelerated because the mobility of the packaged DNA is increased (electrostatic repulsion is reduced). Here we package at elevated temperatures to see whether packaging in phage phi29 is also affected by this solid-to-fluid-like transition.

The temperature of the solid to fluid transition decreases with decreasing ionic screening. The transition temperature is 37 °C with 10 mM MgCl₂ and 28 °C with 10 mM MgSO₄. The transition temperature with 10 mM MgSO₄ is lower because the repulsion energy between DNA strands is increased due to decreased ionic screening of the DNA phosphate.
backbone [21]. At temperatures up to 40 °C and with a Spermine$^{4+}$ concentration that is sufficient to induce DNA condensation, there is no measured solid-to-fluid-like transition. With high Spermine$^{4+}$, the repulsion energy is lower than the energy with repulsive DNA-DNA interactions (10 mM MgCl$_2$ and 10 mM MgSO$_4$) because the net DNA charge is further reduced by Spermine$^{4+}$ ions and the DNA-DNA interaction contains an attractive term. Our ejection study (Chapter 7) strongly suggests that the transition temperature with our phi29 buffer (0.5x TMS) should be lower than the transition temperature with 10 mM MgSO$_4$ (28 °C). We find that the ejection force with 10 mM MgSO$_4$ is lower than with 0.5x TMS at a fixed osmotic pressure. A lower ejection force implies that there is increased ionic screening of the DNA backbone.

**Results**

In a typical packaging measurement, one trapped microsphere is coated with viruses and the other trapped microsphere is coated with DNA molecules. Bringing the two microspheres in close contact initiates packaging (Figure 1A) [22]. To alter the temperature during an optical tweezer packaging experiment, we installed custom copper collars onto our microscope objectives. We heated and cooled the metal collars by connecting them to a temperature controlled water bath (Figure 1B). The flowchamber is sandwiched between both objectives and is in thermal contact with them because there is a water meniscus between the objective and flowchamber that is less than ~1 mm in length. Therefore, the temperature of the objectives is approximately equal to the temperature of the flowchamber. In fact, a separate study used this method to study the dynamics of single DNA molecules at different temperatures, and confirmed that the temperature of the flowcell is equal the temperature of the metal collars [23]. Laser heating of the trapped microsphere contributes an additional ~2 °C to the temperature during packaging [23]. The temperature range, at which packaging measurements were conducted, was maintained between 10 °C and 38 °C.
The Arrhenius equation describes the temperature dependence of chemical reaction rates. It is expressed as, 
\[ r \sim \exp\left(-\frac{E_a}{kT}\right), \]
where \( r \) is the reaction rate (1/s), \( E_a \) is the activation energy, \( k \) is the Boltzmann constant and \( T \) is the temperature. The activation energy \( (E_a) \) is the minimum energy that must be available to a system for a chemical reaction to proceed. In biological systems, the activation energy is typically reduced through the action of enzymes that serve as catalysts. The velocity of the phi29 motor, which is an enzyme, can be characterized using this Arrhenius equation. The activation energy is replaced by the work conducted by the motor, \( F\Delta x \) where \( F \) is equal to 5 pN because we applied a constant 5 pN load to the motor and \( \Delta x \) is a distance parameter that describes the kinetics of ATP hydrolysis. The formula for the equation is then \( \exp\left(-\frac{F\Delta x}{kT}\right) \). This type of temperature dependence has been confirmed with other ATPase molecular motors such as myosin and kinesin [24,25]. A fit to the data yields a \( \Delta x \) value of 0.15 nm, which is very close to a previous estimate (0.11 nm) that was measured by conducting packaging measurements at reduced ATP concentrations (Figure 2) [2].

Increasing the temperature to 38 °C increases the velocity to 470 bp/s while decreasing the temperature to 10 °C decreases the motor velocity to 62 bp/s (Figure 3A). At all temperatures probed, there is no change in the heterogeneity in the length of DNA packaged and no change in the fraction of complexes that pre-maturely stall. This finding indicates that changing the temperature from 10 °C to 38 °C does not inhibit packaging.

On the other hand, we find that decreasing the temperature causes the normalized velocity to be less than the standard condition at high filling levels (Figure 3B). We characterize this reduction in normalized velocity with increasing filling by calculating the area of the normalized vs. filling curve. To compare the reduction in normalized velocities between the measurements taken at different temperatures, we divide the areas to calculate a ratio. Between 0-50% fillings, the ratio of normalized velocity between the imposed
temperature changes and room temperature ($v_{RT}/v_{RT}$) is less than 4%. Above 50% filling, the ratio increases with increasing temperature and decreases with decreasing temperature. Notably, the ratio at 38 °C is 10% larger than standard above 50% filling. At 10 °C, the ratio is 21% smaller than standard above 50% filling. A reduced ratio is presumably due to the packaged DNA forming a conformation that presents higher resistance forces to the motor [26]. However, it should be re-stated that this increase in deceleration with 10 °C does not mean that packaging is inhibited. We find no increase in motor stalling at 10 °C. These findings only suggest that decreasing the temperature drives the system slightly further out of equilibrium but not to a point that stalls packaging. Our finding that increasing the temperature reduces the deceleration in normalized velocity is consistent with the findings of a recent study [9]; a solid-to-fluid-like transition increases the DNA mobility at high temperature. However, reference 9 would most likely predict a larger change in deceleration than what we are finding.

We also conducted packaging measurements at 38 °C and with a reduced motor velocity (Figure 3B, green curve). To lower the velocity to a level similar to the velocity at room temperature (~177 bp/s), we reduced the ATP concentration by 9-fold (50 µM). We hypothesized that increasing the temperature while keeping the velocity fixed could enhance packaging by two mechanisms. One mechanism is that increasing the temperature increases DNA mobility as suggested by Reference 15 and another mechanism is that the packaged DNA has more time to relax because the rate of DNA entering the prohead is reduced. Compared to the normalized room temperature velocity versus filling curve (black curve in Figure 3), the normalized velocity is increased across all filling levels (Fig. 3B).

We realized after we had conducted the low ATP measurements that our interpretation of the low ATP data may be complicated by the allosteric down-regulation mechanism (Ch. 6). The binding of an ATP molecule by a motor subunit is clearly not the rate limiting step at
saturating ATP levels. Once the ATP is bound to a subunit, it is hypothesized that the subunit will transition to a new kinetic state where the ATP and subunit are tightly bound [27]. In this tightly bound state, ATP hydrolysis followed by DNA translocation is extremely favorable. The allosteric interaction slows the packaging rate by reducing the tight ATP binding rate [5]. However, if the ATP concentration is reduced, then the rate limiting step is the ATP binding rate (not tight binding rate where ATP is already bound or in close proximity to a motor subunit). Under these conditions, the motor velocity may no longer serve as an accurate sensor for the DNA conformation.

We also probed for how temperature affects the force generation of the motor. We conducted packaging measurements at temperatures of 16 °C, 24 °C and 32 °C, and with applied forces that ranged between 5 and 50 pN. The velocity monotonically decreases with increasing load, which is consistent with previous measurements that were taken at room temperature [2,22,28]. All three normalized velocity versus force curves lie roughly on top of one another (Figure 4). We are currently writing code for carrying out stochastic stimulations under similar conditions that is based on a recently proposed model for phi29 packaging [29-31]. The model is based on high resolution packaging measurements. When we are finished with the simulations, we will compare the simulation data against the experimental data to see if there is agreement. A separate study carried out stochastic simulations at different forces and with reduced ATP concentrations [27]. Using this phi29 model, they carried out stochastic simulations at different forces and with reduced ATP concentrations. Their simulation data showed agreement with their experimental packaging data, which was taken at applied forces that ranged between 5 and 40 pN and at the same reduced ATP concentrations that the simulations were conducted in. According to their model, the motor is composed of five identical sub-units. During packaging, there are three chemical states that each sub-unit can assume: an Apo or empty state, an ATP bound state, and an ADP bound state. DNA
translocation proceeds as the motor transitions from the ATP to ADP state. During this transition, four of the five sub-units each translocate the DNA by 2.5 bp, which results in a net 10 bp motor step size. Under saturating ATP conditions, an increase in applied force slows the motor by lengthening the transition from the ATP state to ADP state ("burst" phase). A decrease in temperature also slows the motor by lengthening this transition.
Figure 12.1 Diagram of Temperature Collars on Objectives. (A) Initiation of packaging with optical tweezers. (B) We installed copper collars onto our objectives which allowed us to control the temperature during packaging measurements.
Figure 12.2 Velocity vs. Temperature. Velocity as a function of temperature. The red line is an exponential fit to the data (blue line).
Figure 12.3 Velocity vs. Filling between 10 °C to 38 °C. (A) Velocity versus filling at applied temperatures between 10 °C to 38 °C with a 5 pN load. (B) Normalized velocity ($v/v_{\text{max}}$) versus filling. The green line in (B) represents measurements that were conducted at 38 °C and with 50 µM ATP.
Figure 12.4 Velocity vs. Force between 16 °C to 32 °C. (A) Velocity versus applied force at temperatures of 16 °C (blue), 24 °C (black) and 32 °C (red). (B) Normalized velocity versus force.
References


Chapter 13

Evidence for an Electrostatic Mechanism of Force Generation by the Bacteriophage T4 DNA Packaging Motor
Abstract

How viral packaging motors generate enormous forces to translocate DNA into viral capsids remains unknown. Recent structural studies of the bacteriophage T4 packaging motor have led to a proposed mechanism wherein the gp17 motor protein translocates DNA by transitioning between extended and compact states, orchestrated by electrostatic interactions between complimentarily charged residues across the interface between the N- and C-terminal subdomains. Here, we show that site-directed alterations in these residues cause force dependent impairments of motor function including lower translocation velocity, lower stall force, and higher frequency of pauses and slips. We further show that the measured impairments correlate with computed changes in free energy differences between the two states. These findings support the proposed structural mechanism and further suggest an energy landscape model of motor activity that couples the free energy profile of motor conformational states with that of the ATP hydrolysis cycle.

Introduction

Any double-stranded DNA viruses utilize a powerful molecular motor during assembly to translocate DNA into a preformed capsid shell\textsuperscript{1-6}. The motor must do significant mechanical work against forces arising from DNA bending rigidity, electrostatic self-repulsion, and entropy loss, which oppose DNA confinement\textsuperscript{7-12}. Measurements of DNA packaging with optical tweezers in bacteriophages $\phi$29, $\lambda$, and T4 show that all three motors can generate forces $>$50 pN, which is $>$20\times higher than forces generated by skeletal muscle myosin motors\textsuperscript{1,13,14}. The motors differ in DNA translocation rates, which range from up to $\sim$200 bp/s for $\phi$29 to up to $\sim$2000 bp/s for the T4 motor\textsuperscript{13-15}.

A number of studies have begun to examine the microscopic details of the motor mechanism. Measurements of single-DNA packaging dynamics of $\phi$29 via optical tweezers have enumerated the mechanical and chemical kinetic transitions and rate constants, step size,
and coordination of motor subunits\textsuperscript{1,16-18}. Bioinformatics analyses and mutagenesis studies altering the \( \lambda \) and T4 motor proteins indicate the presence of several functional motifs that are homologous with the ASCE (additional strand, conserved E) superfamily of ATPases, including the DNA and RNA translocating helicases and chromosome segregating transporters\textsuperscript{19-21}. Single-molecule packaging measurements with phage \( \lambda \) showed that mutations in the Walker A, C-motif, Q-motif, and other structurally conserved regions of the large terminase subunit cause phenotypic impairments in motor function, including reduced translocation velocity, increased pausing and slipping, and steepening of the force-velocity relationship\textsuperscript{22,23}. The structure-function relationships for these motors have remained unclear due to lack of high-resolution structures, unlike the situation for other biomotors such as helicases and F1-ATPase where sophisticated structural models have been proposed\textsuperscript{24-26}.

The recent determination of a high-resolution X-ray structure of the T4 large terminase subunit (gp17), along with cryo-electron microscopy (EM) reconstruction of the entire prohead-motor complex, has made this system attractive for probing detailed structure-function relationships of viral DNA packaging\textsuperscript{27}. The cryo-EM data indicates that the motor consists of a pentameric ring of gp17 subunits attached to a dodecameric ring of gp20 proteins, together forming the portal entry channel (\textbf{Fig. 1a}). gp17 was found to have globular N- and C-terminal subdomains connected by a flexible linker region. While there has been some debate regarding which subdomain binds to the portal based on studies of several different phages using multiple different techniques\textsuperscript{28-30}, fitting of the gp17 crystal structure into the cryo-EM density of the prohead-motor complex suggests that it is the N-terminal subdomain\textsuperscript{27}. Comparisons also revealed two distinct conformations: a “compact” state seen in the X-ray structure of the gp17 monomer in which the N- and C-terminal domains are in close contact, and an “extended” state, seen in the cryo-EM structure of packaging complexes in
which the N- and C-terminal domains are separated by ~7 Å (previously referred to as “tensed” and “relaxed” states, respectively) \( ^{27} \).

Based on the two different conformations of gp17 seen in the structural data, Sun et al.\(^ {27} \) proposed a model for motor operation in which the DNA is translocated in ~7 Å (~2 bp) increments by the transition of gp17 from the extended to compact state (Fig. 1b). Inspection of the interface between the domains revealed five aligned pairs of complimentarily charged residues, which led to the proposal that this transition is driven by electrostatic interactions between these charged residues across the interface that generate force to translocate the DNA into the prohead. ATP hydrolysis was proposed to regulate DNA interaction and provide energy to return gp17 to the extended state after each 2 bp DNA translocation step. We refer to this model as the “electrostatic force model”. Another model for T4 packaging in which the portal grips the DNA and gp17 exerts force that compresses the DNA until the portal releases it has been proposed\(^ {31} \). Although many aspects of these models are not mutually exclusive, and several different packaging models describing other systems, including one based on the crystal structure of phage Sf6 large terminase gp2, have been proposed\(^ {3,4,32-36} \), the model proposed by Sun et al. is the only one to propose a detailed mechanism implicating specific residues as critical for force generation based on the atomic structure of the motor protein.

Here, we examine the force-generating mechanism of the T4 motor by measurements of single DNA molecule packaging dynamics with increasing applied forces (Fig. 1c) and characterize the effects of mutations that alter charged residues at the interface between the N- and C-terminal subdomains identified in the crystal structure. We also carried out molecular dynamics simulations to predict free-energy difference between the extended and compact states of gp17 and effect of the residue changes. We find that predicted changes in free-energy correlate strongly with measured impairments of motor function, consistent with the electrostatic force model. Our findings suggest a simple mechanochemical energy landscape
picture of gp17 motor dynamics that can explain the experimental observations and simulations.

**Results**

**Site directed mutagenesis**

To investigate whether the pairs of apparently aligned, complimentarily charged residues within the interface domain observed in the gp17 crystal structure are critical to motor function, we used site-directed mutagenesis to alter these residues. **Fig. 2a** shows the locations of the mutated residues in the motor (see **Supplementary Fig. 1** for additional views). We substituted these amino acids with ones with reversed charges (*i.e.*, we changed + to −, or − to +) to weaken the putative attractive electrostatic interactions between the N- and C-terminal subdomains proposed to generate force. In particular, six mutants with single residue changes (K23E, E303K, K305D, E309K, K504E, and E537K) and three with double residue changes (E303K-K504E, E537K-K305D, and E537K-K504E) were studied. Of these, six exhibited little or no packaging activity, as measured in bulk packaging reactions by DNase protection assays (**Supplementary Fig. 2**). While these findings suggest that these residues play an important role in packaging it is important to note that packaging activity as assessed by only these bulk assays can exhibit impairment for multiple reasons, including failure of motor proteins to fold assemble into active multimeric complexes, or initiate interactions with DNA. Three of the mutants (E303K, E537K, and E537K-K305D), exhibited sufficient activity to permit detailed measurements of packaging dynamics using the single-molecule optical tweezers assay.

**Measurements of DNA packaging dynamics at low force**

We first carried out single-molecule packaging measurements using the low force clamp technique described previously. Briefly, T4 prohead-motor complexes were assembled using purified recombinant gp17, attached to microspheres via antibodies, injected
into a fluid chamber containing ATP, and trapped in one optical trap. DNA molecules were biotinylated at one end and attached to streptavidin-coated microspheres and trapped in a second optical trap. Packaging was initiated by bringing the two microspheres into proximity such that the motor binds to and begins reeling in the DNA, thus exerting a force that pulls the two microspheres together (Fig. 1c). DNA translocation was tracked using a feedback control system that held the applied load force at a constant value of 5 pN, which is low compared to the maximum force generated by the WT motor (>60 pN).

Measurements were made with the three mutants and with wild-type (WT) gp17 for comparison (Fig. 2b). We found that E537K mutants packaged at nearly the same rate (~600 bp/s) on average as WT, while E303K and E537K-K305D mutants showed a modest reduction to ~400 bp/s. The reductions were partly due to an increase in pausing and slipping and partly due to inherently reduced motor velocity (as shown below). These results clearly show that some of the charged residues at the interface play an important role in the DNA translocating function of the T4 motor. However, given that one mutant (E537K) exhibited no measurable change, the other two mutants exhibited only modest changes, and unexpectedly, the double mutant (E537K-K305D) did not show greater impairment, support for the electrostatic force model from these measurements alone could be considered ambiguous.

High force measurements

We next investigated the motor response under increasing applied loads, where the impact of changes in residues putatively involved in force generation would be expected to be greater. In our previous studies of the WT motor, high force measurements were made by holding the trap positions fixed and allowing the tension in the DNA to build up due to the shortening of the DNA tether accompanying packaging\textsuperscript{14}. This approach is undesirable for characterizing mutants because they package DNA at slower rates, which would cause a slower build-up of force. This would lead to a bias in the comparison of the WT and different,
as different complexes would experience different force loading histories. Also, prolonged force build up frequently leads to rapid detachment of the tethered complexes before the target force is reached, due to the DNA slipping out of the prohead or dissociation of the prohead from the antibody-coated microsphere\textsuperscript{14,37}. Therefore, in the present work we implemented a new protocol in which we first packaged ~5 kbp of DNA at low force (5 pN) to confirm activity, and then quickly ramped the force to a higher value and clamped it via feedback control to measure the response of the motor. Thus, all complexes, WT and mutants, were subject to the same force loading history.

Using this new protocol, force clamp measurements were recorded at 5, 15, 30, 40, 50, and 60 pN loads. Examples of individual packaging traces are shown in Fig. 2c,d, highlighting the highly variable and stochastic nature of packaging by the gp17 motor. We also note the presence of pauses in translocation (flat regions) and slippage (negative slope) interspersed with DNA packaging episodes (positive slope). The overall trend is that velocity decreases with increasing applied load, which implies that the rate limiting step in the mechanochemical cycle involves DNA translocation and, hence, mechanical work done by the motor against the load slows down the overall motor velocity\textsuperscript{38}. Mutations further slow packaging and the time spent pausing and slipping also increases with increasing load and mutations. A perhaps unexpected minor feature in the data is the occurrence a similar average packaging rate at 30 pN and 15 pN and slightly higher % time packaging at 30 pN than at 15 pN. The cause of this behavior is not clear, but it may be related with the observation that the motors sustain the 15 pN load for much longer time (115 sec, on average) than the 30 pN load (32 sec, on average).

We analyzed ensembles of packaging trajectories to determine several averaged metrics of motor function (Fig. 3). Specifically, we calculated the “packaging rate”, defined as
the net length of DNA packaged per unit time; the “motor velocity”, defined as the rate of DNA translocation during episodes of packaging (i.e., not including pauses and slips); and the “percent time spent packaging”, defined as the percentage of time packaging occurs (vs. pausing or slipping). All three metrics show that the residue changes cause more severe impairment at high load than at low load. For example, the packaging rate for mutant E303K, which was ~400 bp/s at 5 pN, drops below zero at forces ≥15 pN. E303K exhibits episodes of packaging, but slips so frequently that there is no net packaging. The packaging rates for E303K and E537K-K305D, which were nearly the same at 5 pN, also exhibited a clear difference at forces ≥15 pN. The rate for E537K, which is the same as that of WT at 5 pN, drops below zero at 50 pN whereas the wildtype averages ~20 bp/s. Above 30 pN, the percent time packaging for all the mutants drops clearly below that of WT, and at 60 pN it drops to 0% for all the mutants vs. ~12% for WT. At 50 pN the mutants displayed only brief episodes of highly slowed packaging, and at 60 pN no packaging was detected, whereas the WT motor was still able to package with an average motor velocity of ~100 bp/s.

Thus, the mutant motors exhibit a reduced force-generation capacity, with $F_{\text{max}}$ between 50 and 60 pN. Overall, these results showed that all of the charged residues altered by mutagenesis play an important role in motor function. These results are consistent with the electrostatic force model because residue changes expected to weaken the electrostatic interactions proposed to drive the extended-to-compact transition indeed cause impairments in the packaging rate and force-generating capacity of the motor.

**Molecular dynamics-based free energy calculations**

We next sought to investigate more quantitatively how different residue changes would be expected to affect the conformational change of gp17 from the extended to compact states. In particular, we investigated whether the predicted degree of impairment would follow a similar trend as observed in our experimental measurements of impaired function, which
increased in the following order: WT, E537K-K305D, E537K, and E303K. To this end, we carried out molecular dynamics (MD) simulations of gp17 to predict the free energy difference $\Delta G_{EC}$ between the extended (E) and compact (C) states observed in the structural studies. Because of the limited structural information for this system, we do not attempt to model the motor-DNA interaction or the full ATP hydrolysis cycle. Thus, our modeling is necessarily more limited compared with the more intricate models that have been developed to understand detailed structure-function relationships for other biomotors such as the large tumor antigen helicase and F1-ATPase, where structures are available in different nucleotide states and for the helicase with DNA bound.  

We determined $\Delta G_{EC} = G_C - G_E$ by computing the free energies of the compact ($G_C$) and extended states ($G_E$) relative to a common reference state. The relative free energies for each state were calculated as “binding free energies” between the N- and C-terminal domains using the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) approach. In these calculations, the compact state was modeled as the available crystal structure of gp17 in the compact state while the extended state observed in cryo-EM studies was modeled from the crystal structure by imposing a 7Å separation between the N- and C-terminal domains.

Our calculations yielded $\Delta G_{EC} = -38.21\pm4.42$ kcal/mol for WT gp17, $-19.95\pm9.99$ kcal/mol for E537K-K305D, $-6.14\pm3.44$ kcal/mol for E537K, and $-2.79\pm5.55$ kcal/mol for E303K. These values do not include contributions from changes in the configurational (mostly vibrational) entropy of the gp17, as we found that estimates of vibrational entropy from normal mode calculations yielded large uncertainties. We do not, however, expect vibrational entropy to vary significantly across different mutants. Notably the determined free energy values are all negative, consistent with the transition from the extended to compact state being most favorable for the WT gp17 and favorable to a lesser degree for the mutants. We also carried out calculations for the mutant E309K, which
exhibited no packaging activity in both bulk packaging assays and optical tweezers assays. Notably for this mutant we calculated a positive or near zero (unfavorable) $\Delta G_{EC} = 1.83 \pm 10.3$ kcal/mol.

For WT gp17, the calculated absolute value $|\Delta G_{EC}| \approx 38$ kcal/mol is equivalent to $\sim 260$ pN-nm of energy, which implies that the maximum force that can be exerted during the $\Delta x = 0.7$ nm separation change between the N- and C-terminal subdomains is $F_{\text{max}} = |\Delta G_{EC}|/\Delta x \approx 370$ pN. Thus, our calculations show that the extended-to-collapsed state transition can indeed produce more than enough force to explain the very high measured forces (>60 pN) generated during packaging. The energy released by hydrolysis of one ATP under the experimental conditions is also sufficient to generate 60 pN of force, as discussed previously. However, the predicted $|\Delta G_{EC}|$ is about twice the $\sim 130$ pN-nm free energy released by hydrolysis of a single ATP molecule, suggesting hydrolysis would not provide enough energy to return from the compact state to the extended state. However, several caveats regarding the calculations could potentially be responsible for this discrepancy. Due to lack of detailed structural information, we approximate the extended state structure by simply displacing the center-of-mass of the C-terminal domain away from that of the N-terminal domain by 7 Å. In reality, changes in orientations of the domains may affect $\Delta G_{EC}$ values. Also, our calculations ignore the other components of the motor complex—neighboring gp17 subunits along with the threaded DNA molecule—whose configuration remains undetermined. These components likely occlude portions of the N- and C-terminal domain interface from getting solvated, causing our calculations to either over- or under-predict the solvation free energy. Our calculations neglect potential contributions from the configurational entropy of gp17 within the full pentameric active complex, as discussed above. Finally, the MM-GBSA approach itself contains several approximations which may introduce inaccuracies in the
absolute free energies values⁴¹, and, like all other biomolecular simulations, our calculations are also subject to the inherent inaccuracies in the force field representation of protein, solvent, and ions. For these reasons, we expect the relative trend of changes in \( \Delta G_{EC} \) with residue changes to be more meaningful than absolute \( \Delta G_{EC} \) values.

**Correlation of measured and computed impairments**

To compare the measured and calculated metrics of motor function we calculated the Pearson correlation coefficients (0 = “no correlation”, 1 = “perfect correlation”) between each of the three measured quantities (Fig. 3, right panel), at each of the five increasing values of applied force, and the calculated \( \Delta G_{EC} \) values for the WT gp17 and three mutants. We observe significant correlations in all cases and the degree of correlation increases with increasing applied force. At 5 pN, the packaging rate and motor velocity correlate only weakly with \( \Delta G_{EC} \), but the correlation coefficients increase to 0.88 and 0.96, respectively, at 50 pN. The correlation coefficient for time spent packaging also rises from 0.65 to 0.95 as the force is increased from 5 to 50 pN.

Thus, we have shown that the measured degree of motor impairment due to the residue changes correlates well with the calculated degree of impairment in terms of change in free-energy difference between the extended and compact states of gp17. The strength of correlation increases at high force, where the motor is subject to higher load, consistent with the proposal that the E-C transition is responsible for force generation. Several features, such as the double mutant E537K-K305D exhibiting less impairment than single mutant E303K, are reconciled by these calculations. The measured % times spent packaging and motor velocities also asymptote towards zero with increasing force in the order E303K, E537K, E537K-K305D, WT, consistent with the order of increasing magnitudes of the calculated favorable free-energy changes.

**Mechanochemical energy landscape model**
Based on our findings above, we propose a minimal free energy landscape model of gp17 mechanochemistry (Fig. 4) that provides additional insights into the dynamics of the phage T4 DNA packaging motor. As discussed before, we do not attempt to model the motor-DNA interactions because of the lack of structural information. In this model, the free energy landscape \( G_{\text{tot}} \) governing gp17 motor function under applied loads is made up of contributions from the intrinsic conformational free energy landscape of gp17 \( G_{\text{gp17}} \), the chemical free energy of ATP during the hydrolysis cycle \( G_{\text{ATP}} \), and the mechanical work performed by gp17 against the applied load during DNA translocation \( W \), i.e.,

\[
G_{\text{tot}} = G_{\text{gp17}} + G_{\text{ATP}} + W.
\]

We consider the simplest “tight coupling” model in which ATP hydrolysis is assumed to result in one DNA translocation step. The energetics of ATP hydrolysis by motors with structurally homologous ATPase domains has been well characterized and has been shown to consist of two main steps that release energy: the ATP binding and product release steps\(^{24}\). The approximate \( G_{\text{ATP}} \) profile based on Ref. \(^{24}\) is sketched as the blue line in Fig. 4b-d; in these conditions, the ATP binding and product release steps yield \( \sim 15 k_B T \) and \( \sim 10 k_B T \) energies, respectively.

We propose a minimal model consisting of three conformational states of gp17 (Fig. 4a). Before ATP binding, gp17 is in an Apo state \((A)\) whose structure is not known. In the first step, ATP binds and provides the energy to drive the transition from the Apo state to the extended state \((E)\), whereupon it binds to the DNA. In the second step, ATP is hydrolyzed to ADP and \( P_i \) and gp17 transitions from the extended state to the compact state \((C)\), and translocates the DNA by 2 bp. In the third step, ADP and \( P_i \) are released and the DNA is passed onto the next gp17 subunit in the motor ring (alternatively, \( P_i \) could be released earlier during the second step, as suggested by studies of the phage \( \Phi 29 \) motor\(^{17}\)). The original gp17 subunit goes back to the \( A \) state. This order of conformational-chemical transitions allows for
the $E \to C$ transition—that goes “downhill” in free energy, as observed from our free energy calculations, and requires no external energy—to occur during the ATP hydrolysis step that itself releases little or no energy$^{24}$. This then allows for the ATP binding and product release steps, the two energy-producing chemical transitions, to be utilized for pushing gp17 towards the energetically unfavorable extended state ($A \to E$) and releasing gp17 from its energetically favorable compact state ($C \to A$), respectively. This proposed $G_{gp17}$ profile is shown in Fig. 4b-d as the red line. Note that based on our simulations we only know that $G_{gp17}$ decreases during the $E \to C$ transition, with free energy difference

$$\Delta G_{EC} = G_{gp17}(C) - G_{gp17}(E) < 0,$$

and increases during the $A \to E$ and $C \to A$ transitions, possibly involving energy barriers, but the magnitudes of the free energy differences and the heights and locations of the energy barriers are not known.

The mechanical work $W$ is given by the applied force $F$ multiplied by the distance the DNA is translocated against the force. During the $A \to E$ transition, $W = 0$, as there is no DNA packaging during this transition. During the $E \to C$ transition, $W = F \cdot x$, where $x$ is the distance the DNA moves during this transition. At the end of this transition, the net amount of work performed is $W = F \cdot \Delta x$, where $\Delta x = 2$ bp. No additional work is performed during the $C \to A$ transition, and the work remains at $W = F \cdot \Delta x$. The resulting $W$ profile is shown as the green line in Fig. 4b-d.

In the absence of a load force (Fig. 4b), the overall free energy profile $G_{tot}$ (shown as the black line) exhibits a largely downhill path, potentially with energy barriers between each of the three conformational transitions, which facilitates efficient packaging of DNA. As described earlier, this is made possible by the constructive coupling between conformational and chemical transitions, which allows for efficient utilization of the energy released from ATP hydrolysis. Overall, each DNA translocation step of 2 bp leads to a net reduction in the
free energy of the system (gp17 + solution) equal to the total free energy released by the hydrolysis of one ATP molecule, i.e., $\Delta G_{ATP} \approx 25 k_B T$.

The observed reduction in the motor velocity with increasing load force $F$ (Fig. 3b) suggests the presence of an energy barrier between the E and C states. The applied force, due to the mechanical work $W$, increases this barrier height by an amount $\sim F \cdot \Delta x^*$, where $\Delta x^*$ is the location of the barrier relative to the E state along the force direction. Assuming an Arrhenius-like dependence in transition rates across barriers, the DNA translocation rate $k_t$ (and hence the motor velocity) should then approximately decrease with increasing $F$ via $k_t \propto \exp\left[-(\Delta G^* + F \cdot \Delta x^*)/k_B T\right]$, where $\Delta G^*$ denotes height of this energy barrier (Fig. 4c).

However, based on our findings with gp17 mutants we propose that at sufficiently large $F$, the work term tilts the energy landscape between E and C states to such a large extent that the free energy of state C now becomes higher than that at the original barrier, making the crossing of state C the rate limiting step (Fig. 4d). The relevant barrier height then becomes equal to the free energy difference between states E and C, as given by $\Delta G_{EC} + F \cdot \Delta x$. The rate of DNA translocation should consequently reduce via $k_t \propto \exp\left[-(\Delta G_{EC} + F \cdot \Delta x)/k_B T\right]$.

Our measurements show that the mutant motors generate smaller packaging forces than WT motors. This effect can be understood based on our free-energy calculations, which predict the E $\rightarrow$ C transition as being less favorable for mutants, leading to larger state C free energies for the mutants as compared to the WT motor, as depicted in Fig. 4b-d. In addition to having lower stall forces (<60 pN), the mutants also exhibit slower rates of packaging as compared to WT motors, and this difference becomes more significant at high loads. This can be explained by our model, which predicts that the rate of DNA translocation varies as $k_t \propto \exp\left[-(\Delta G^* + F \cdot \Delta x^*)/k_B T\right]$ at low loads and as $k_t \propto \exp\left[-(\Delta G_{EC} + F \cdot \Delta x)/k_B T\right]$ at high loads. That the mutants and WT motors exhibit smaller differences at low forces implies that they
both exhibit similar energy barriers $\Delta G^\ddagger$ (Fig. 4c). In contrast, the stronger effect of mutations on the packaging rate at high loads is consistent with the mutations decreasing the magnitude of $\Delta G_{EC}$ (Fig. 4d).

The observed increase in pausing and slipping of DNA during translocation with high applied force and with mutations (Fig. 2) can also be explained by the energy landscape model. A recent study of T4 packaging at low ATP concentration suggested the presence of a pause-“unpackaging” state in the mechanochemical kinetic cycle that branches off from the DNA translocation pathway$^{42}$. Our present results further suggest that with saturating ATP the motor can switch to this alternate pathway while residing at the E state under a high load force, which represents the most stretched conformation of the motor (Fig. 4a). It then follows that conditions slowing down the E $\rightarrow$ C transition rate, such as the applied load and the residue changes studied here, would increase the time that the motor resides in the E state. This in turn increases the chances of the motor entering the proposed pause-unpackaging state, leading to increased rates of pausing and slipping, which is consistent with our observations.

**Discussion**

Using a combination of single-molecule measurements with applied forces, site-directed mutagenesis, and molecular dynamics simulations, we have quantitatively demonstrated the importance of electrostatic interactions between residues located at the interface of the N- and C-terminal subdomains of gp17 to mediate DNA translocation. These findings support the T4 motor force-generation mechanism proposed by Sun et al. on the basis of structural data$^{27}$. We show that our results with both WT and mutant motors under a wide range of load forces can be rationalized, at least qualitatively, by an energy landscape model that couples the free energy profile of motor conformational states with that of the ATP hydrolysis cycle.
Many molecular motors rely on large-scale structural rearrangements to forcibly translocate cargo. These rearrangements are often coupled to structural changes in the vicinity of the ATP binding pocket during the ATP hydrolysis cycle, which cause allosteric effects that may be leveraged to produce large-scale motions. Although coordination of multiple parallel electrostatic interactions across an interface between two subdomains has not been previously demonstrated as a mechanism to directly produce motor force, evidence suggests that both myosin and FOF1-ATP synthase rely on electrostatic interactions between single pairs of charged residues to initiate structural transitions largely mediated by polar and hydrophobic interactions. As the T4 motor shares some features with many other ring-shaped nucleoside triphosphatases, including enzyme complexes involved in DNA replication and transcription (helicases), protein degradation, and chromosome segregation, the force generation mechanism investigated here may well have broader implications.

**Methods**

**Materials.**

A 25,339 bp dsDNA construct used as a substrate for packaging was prepared by PCR from lambda phage DNA (NEB, Inc.) using primers biotin-5'-CTGATGAGTTCGTGTCGTCGTAATC and 5'-ATCCGATCTGCGTTACCGAATGGATGGATG (Operon, Inc.) and the PCR Extender System (5 Prime, Inc.).

T4 phage heads were purified from the 10am.13am.hoc-del.soc-del phage mutant (E. coli P301 (sup-) cells infected with this mutant were lysed in Pi-Mg buffer (26 mM Na2HPO4, 68 mM NaCl, 22 mM KH2PO4, 1mM MgSO4, pH 7.5) containing 10 µg/ml DNase I and chloroform and incubated at 37°C for 30 min. The lysate was subjected to two low (6,000 x g for 10 min) and high (35,000 x g for 45min) speed centrifugations and the final heads pellet was resuspended in Tris-Mg buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl and 5 mM MgCl2) and purified by CsCl density gradient centrifugation. The major head band
sedimented at about 1/3rd from the bottom of a 5 ml gradient was extracted and dialyzed overnight against Tris-Mg buffer. The heads were further purified by DEAE-Sepharose ion-exchange chromatography. The peak heads fractions were concentrated and stored at -80°C.

Wild-type and mutant gp17 motor proteins were purified from *E. coli* BL21 (DE3) RIPL cells harboring the recombinant clones\(^{46,47}\). The recombinant proteins were over-expressed with 1 mM IPTG for 2 h at 30°C. The cells were harvested by centrifugation (4,000 x g for 15 min at 4°C) and resuspended in HisTrap binding buffer (50 mM Tris HCl, pH 8.0, 20 mM imidazole, and 300 mM NaCl). The cells were lysed using French-press (Aminco, USA) and the soluble fraction containing the His-tagged fusion protein was isolated by centrifugation at 34,000 x g for 20 min. The supernatant was loaded onto a HisTrap column (GE Healthcare), washed with 50 mM imidazole containing buffer and the protein was eluted with 20-500 mM linear imidazole gradient. The peak fractions were concentrated and purified by size exclusion chromatography using Hi-Load 16/60 Superdex-200 (prep-grade) gel filtration column (GE Healthcare) in a buffer containing 20 mM Tris-HCl, pH 8.0 and 100 mM NaCl. The peak fractions were concentrated and stored at -80°C.

**Bulk DNA packaging assays.**

DNase protection assays shown in Supplemental Fig. 2 were performed as follows\(^{48}\). The purified wild-type or mutant gp17 proteins were incubated with phage heads in the presence of 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 3 mM MgCl\(_2\), 1 mM ATP, and phage λ DNA (48.5 kb) for 30 minutes at 37°C. DNase I (Sigma) was added to terminate DNA packaging and digest the unprotected DNA. Proteinase K (0.5 µg/µl), 50 mM EDTA, and 0.2% SDS were added and incubated for 30 minutes at 65°C to digest DNase I and capsid and release the packaged DNA. The samples were electrophoresed on 0.8 % (w/v) agarose gel for 2-3 h at 100 V and DNA was stained with ethidium bromide.

**Single-molecule packaging measurements.**
T4 head-motor complexes were prepared by mixing 1.7×10^{10} heads with 70 picomols of gp17 in a solution containing 50 mM Tris-HCl pH 7.5, 5 mM MgCl_2, 80 mM NaCl, 1 mM gamma-S-ATP (non-hydrolyzable ATP analog), and 450 ng of 120 bp "initiating" DNA (which we found stabilizes the competent head-motor complex)\textsuperscript{14}. This solution was incubated at room temperature for 45 min. 2 µl of T4 antibody coated 2.2 µm diameter protein-G microspheres (Spherotech) were mixed 10 µl of these stalled T4 complexes and incubated at room temperature for 45 min. The biotinylated dsDNA was attached to 2.2 µm diameter streptavidin coated microspheres (Spherotech). Measurements were carried out in solution containing 50 mM Tris-HCl pH 7.5, 5 mM MgCl_2, 80 mM NaCl, 1 mM ATP, and 0.05 g/L BSA.

**High force measurements.**

Packaging was first measured at low force (5 pN) for 5-10 seconds in force clamp mode\textsuperscript{14}, to verify motor activity. The force was then quickly ramped (within 0.2 s) to a higher value by adjusting the set-point on the force-clamp control system. DNA translocation (packaging and/or slipping) was then tracked in force-clamp mode until the DNA tether was lost. The force was monitored at 2 kHz and was held constant by a feedback control system that adjusts the separation between the two traps in 0.5 nm increments. The optical tweezers system was calibrated as described previously\textsuperscript{49,50}.

**Data Analysis.**

The motor velocity was calculated using methods similar as those described previously\textsuperscript{14} by linear fitting of the DNA length vs. time records in a 1 s sliding window. Slips larger than 100 bp and pauses in which the velocity dropped to <12 bp/s in the 1 s window were not included in determining "motor velocity" (but were included in determining overall "packaging rate").

**Computational Methods**
Overview.

Our aim is to compute the free energy difference \( \Delta G_{EC} \equiv G_C - G_E \) between the extended and compact states of gp17 for the wild type and mutant proteins. In principle, \( \Delta G_{EC} \) can be computed from all-atom molecular dynamics (MD) simulations via approaches like umbrella sampling, though such approaches would entail prohibitive computational costs for systems as large as gp17. To this end, we use the Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) method\textsuperscript{39}, which provides quick estimates of the binding free energy of molecular complexes by approximating the solvation free energy using implicit solvent mean field models. However, the application of MM-GBSA to computing intramolecular binding free energies, as opposed to intermolecular binding free energies, requires the N- and C-terminal domains to be treated as separate “molecules”, which assumes, consistent with structural features observed in Ref. 27 that the hinge joining the two domains is flexible and does not contribute much to the free energy difference between the compact and extended states. One can then obtain \( \Delta G_{EC} \) using the free energy cycle shown in Supplemental Fig. 3, where the MM-GBSA method is used to compute the binding free energies \( \Delta G_{bind,E} \) and \( \Delta G_{bind,C} \) for assembling the N- and C-terminal domains into extended and compact states from a common isolated state in which the two domains do not interact with each other. The required free energy difference can then be simply calculated as \( \Delta G_{EC} = \Delta G_{bind,E} - \Delta G_{bind,C} \).

Chapter 13, in full, is a reprint that the dissertation author was the principal researcher and author of. The material appears in Nature Communications. (N. Keller, AD Migliori, TI Alam, M Mahalingam, VB Rao, G Arya, DE Smith) (2014). Evidence for an electrostatic mechanism of force generation by the bacteriophage T4 DNA packaging motor.
Figure 13.1 Overview of T4 Packaging Motor. Schematic overview of the T4 packaging motor structure and the single-molecule measurement technique. (a) The T4 packaging motor consists of concentric rings of gp17 (motor) and gp20 (portal) proteins at the special five-fold vertex of the viral capsid. (b) Structural data indicates that gp17 can adopt extended (left) and compact states (right). The X-ray structure shows a large interface between the N-terminal (composed of two sub-regions, N-I and N-II) and C-terminal globular subdomains containing pairs of apparently aligned complementarily charged residues. The electrostatic force model proposes that attractive interactions between these residues generates the force during a conformational change from the extended to the compact state that translocates the DNA into the capsid in steps of ~2 bp. (c) Dual optical tweezers are used to measure the packaging of single DNA molecules into single capsid-motor complexes under applied load forces.
Figure 13.2 Force vs. Time Traces of Mutants and Wildtype. Locations of altered charged residues and measurements of DNA packaging dynamics. (a) Open book representation of the N- and C-terminal domains of gp17 (PDB ID code 3CPE) displaying the charged residues studied in this work. Changes in the residues labeled in grey resulted in zero bulk packaging while those labeled in black resulted in some packaging, leading to their further study through optical traps. Red indicates acidic interface residues, while blue indicates basic interface residues. (b-d) Representative measurements of single DNA molecule packaging dynamics (length packaged vs. time) with (b) 5 pN applied load, (c) 30 pN, (d) 50 pN. The colors indicate WT (black), E537K (red), E537K-K305D (green), and E303K (blue) gp17 motors.
Figure 13.3 Experimental vs. Computational Data. Experimental metrics of motor activity and correlation with computed free-energy changes in gp17. (a-c, left panel) Average measured packaging rate (a), motor velocity (b), and % time spent packaging (c) for wild type gp17 (black), E537K (red), E537K-K305D (green), E303K (blue). The number of independent packaging events measured for each complex is given in Supplemental Table 1, and the error bars indicate standard error of the means. (a-c, right panel) corresponding Pearson correlation coefficients (min=0, max=1) between experimental metrics of motor function (adjacent plots) and calculated free energy changes between the extended and compact forms of gp17. The error bars indicate standard error of the means.
Figure 13.4 Mechanochemical Energy Landscape. Mechanochemical energy landscape model inferred from experimental and computational findings. (a) Proposed coupling between conformational transitions in gp17 and chemical transitions in ATP during a 2 bp DNA translocation cycle driven by the hydrolysis of one ATP molecule. The pausing and slippage of DNA is represented as an alternative state branching off from and rejoining at the extended state of gp17. (b-d) The conformational energy landscape of gp17 (red line), the ATP hydrolysis free energy profile (blue line), and mechanical work (green line) add up to yield the overall mechanochemical energy landscapes (black line). The shapes of the different energy landscape are schematically drawn for three cases: zero load (b), small load (c), and large applied loads (d).
References


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