

Elastic Properties and Heterogeneous Stiffness of the Phi29 Motor Connector Channel

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ABSTRACT The DNA packaging motor of the bacteriophage ϕ 29, comprising head-tail connector, ATPase, and pRNA, transports the viral DNA inside the procapsid against pressure differences of up to ~60 atm during replication. Several models for the DNA packaging mechanism have been proposed, which attribute different roles to the connector, and require specific mechanical properties of the connector. To characterize these properties at the atomic level, and to understand how the connector withstands this large pressure, we have carried out molecular dynamics simulations of the whole connector both in equilibrium and under mechanical stress. The simulations revealed a quite heterogeneous distribution of stiff and soft regions, resembling that of typical composite materials that are also optimized to resist mechanical stress. In particular, the conserved middle α -helical region is found to be remarkably stiff, similar only to structural proteins forming viral shell, silk, or collagen. In contrast, large parts of the peripheral interface to the ϕ 29 procapsid turned out to be rather soft. Force probe and umbrella sampling simulations showed that large connector deformations are remarkably reversible, and served to calculate the free energies required for these deformations. In particular, for an untwisting deformation by 12° , as postulated by the untwist-twist model, more than four times' larger energy is required than is available from hydrolysis of one ATP molecule. Combined with previous experiments, this result is incompatible with the untwist-twist model. In contrast, our simulations support the recently proposed one-way revolution model and suggest in structural terms how the connector blocks DNA leakage. In particular, conserved loops at the rim of the central channel, which are in direct contact with the DNA, are found to be rather flexible and tightly anchored to the rigid central region. These findings suggest a check-valve mechanism, with the flexible loops obstructing the channel by interacting with the viral DNA.

INTRODUCTION

The bacteriophage ϕ 29 infects the bacterium *Bacillus subtilis* and uses its molecular machinery for replication (1). During this process, the precursor capsid (procapsid) forms by the incorporation of newly synthesized proteins inside the bacteria. Subsequently, a DNA packaging motor assembles at the base of the procapsid and transports viral DNA into it (1,2). The motor comprises the connector (shown as red in Fig. 1 A), prohead-RNA (pRNA), and an ATPase (3–9). Both the pRNA as well as ATPase detach from the connector after completion of DNA packaging, and the tail proteins of the phage particle join at the bottom region of the connector, completing the formation of new phage particles.

The DNA packaging motor transports viral DNA against an internal-to-external pressure difference of ~60 atm (10) generated by the densely filled viral DNA inside the procapsid. Accordingly, it is one of the strongest biological molecular motors, which renders it a promising choice for a motor in nanodevices (2). Further potential applications have been suggested, such as a molecular sorter in nanopore-based DNA sequencing devices, or as a model system to develop antiviral drugs to treat infections caused by herpes simplex virus, adenovirus, parvovirus, or pox virus (2). Additionally, motor pRNA is used as a carrier for ribozyme and antisense

RNA to inhibit the Hepatitis B virus, which renders it a potential gene delivery system (11).

With a view to these diverse emerging applications, several studies were aimed at elucidating the underlying molecular DNA packaging mechanism, and in particular the properties and the role of the connector (2,12,13). The average viral DNA translocation rate is ~165 basepairs per second at the initial phase, with a gradual decrease as packing progresses (14). The motor generates up to ~110 pN at the final stage of the filling (14). Translocation of 10 basepairs of viral DNA into the procapsid requires hydrolysis of five ATP molecules by the ATPase (13).

Here, we focus on the connector as one of the motor components, for which the crystal structure has been determined (9,15). The connector is a truncated cone-shaped dodecameric protein complex that forms a channel through which viral DNA is transported inside the procapsid (9) (Fig. 1, A and B, see also Fig. S1 in the Supporting Material). The connector can be divided into three major regions—an upper, a middle, and a bottom region (blue, brown, and green regions in Fig. 1 B). The upper and middle regions are in direct contact with the procapsid, whereas the bottom region is connected to the middle region by a hinge region (red) and protrudes toward the outside of the procapsid. The central DNA transport channel is mainly formed by the middle and bottom region.

To uncover the mechanism by which the connector withstands the large pressure difference of up to ~60 atm, we focus on its mechanical properties. Because the connector

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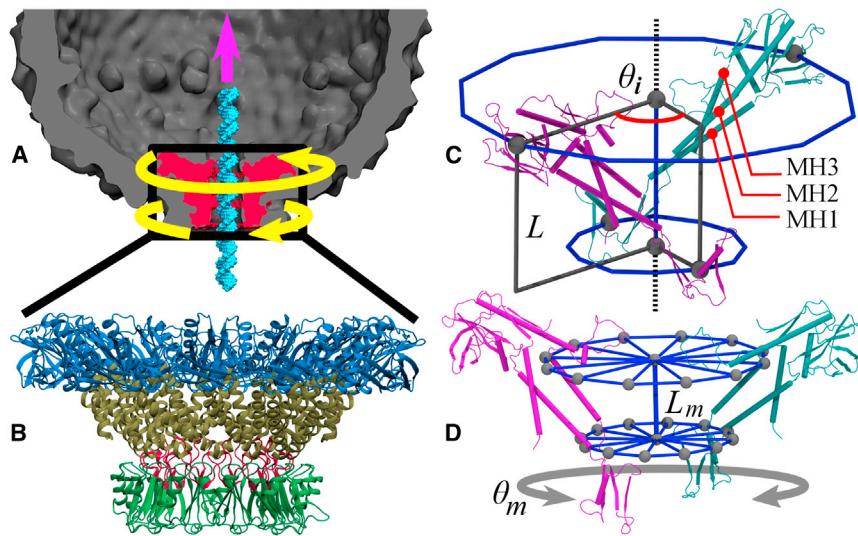
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bottom regions along the channel axis (black dotted line). Twist deformation is defined via relative rotation of the bottom rim with respect to the top rim (blue circles) and determined by the average angle θ of all torsion angles θ_i of the 12 individual subunits MH1, MH2, and MH3, referred to in the text. (D) Similarly, compression and twist deformations of the middle region are characterized by the length L_m between the center (gray spheres) of the upper and the lower disk, and the respective twist angle θ_m . The disks (blue) are defined via the C α atoms of the MH1 and MH2 terminal residues, respectively (gray spheres). The centers of mass of the C α atoms of the respective disk (axial gray spheres).

is part of the procapsid (Fig. 1 A), its mechanical properties should also be similar to those of the procapsid, which have been probed earlier by atomic force microscopy (AFM) (16,17). In particular, a very large elastic modulus of ~ 1.8 GPa (i.e., similar to that of hard plastic) was measured, which was therefore suggested to be crucial for withstanding such high pressure difference (17). For the procapsid, a spring constant of ~ 0.07 N/m has been determined, whereas the connector region was found to be softer, with a spring constant of ~ 0.04 N/m (16). These findings render the question of how such a channel protein resists pressure differences of up to 60 atm even more puzzling.

The role of the connector in the DNA packaging mechanism is also unclear. One model, from Hendrix (18), proposed that the connector performs full rotations with respect to the procapsid, progressing by 12° in each step, thereby translocating two basepairs of the viral DNA inside the procapsid by consuming energy released from the hydrolysis of one ATP. However, more recent single-molecule fluorescence spectroscopic studies performed by Hugel et al. (19) ruled out such complete rotation of the connector.

A second model, which is compatible with the above results, has been proposed by Simpson et al. (9) and assumes partial rotations, with springlike coupled compression-stretching and twisting-untwisting motions (indicated in Fig. 1 A) of the middle helical region. According to this model, which is subsequently referred to as untwist-twist model, the whole connector first untwists by 12° through untwisting of its middle region (yellow arrows) under consumption of one ATP molecule; simultaneously, it stretches by 0.68 nm and grasps the next two basepairs of DNA. The

FIGURE 1 Illustration of the untwist-twist DNA packaging model; different regions of the connector (left), and definition of length and twist deformations (right) used for analysis. (A) The connector (red) and the DNA (blue) are aligned with the cryo-EM density map (obtained from the EM data bank, code EMD-1117 (7,8)) of the ϕ 29 procapsid (gray). According to the untwist-twist DNA packaging model (9), the connector untwists and twists by 12° (yellow arrows) while the DNA is transported in two basepairs steps toward the procapsid interior (magenta arrow). (B) The structure of the connector reveals four distinct regions. The upper (blue) region faces the procapsid interior; the middle (brown) and bottom (green) regions, which form the channel, are connected by the hinge (red) region. (C and D) Two opposite (purple, cyan) out of the 12 connector subunits are illustrated in cartoon representations. (C) The whole connector's compression-stretching motions are characterized by the length L between the centers of mass (dark gray spheres) of the upper and the lower disks. (D) Similarly, compression and twist deformations of the middle region are characterized by the length L_m between the center of mass of the upper disk and the center of mass of the lower disk, and the respective twist angle θ_m . The disks (blue) are defined via the C α atoms of the respective disk (gray spheres).

connector then relaxes by twisting and contracting into its original configuration, thereby generating the force required to translocate two basepairs of the viral DNA toward the interior of the procapsid. Accordingly, this model requires the untwisting of the connector to be coupled by $17.6^\circ/\text{nm}$ to an increase of its length.

A third model, the push-and-roll model, from Yu et al. (12), Moffitt et al. (13), and Aathavan et al. (20), proposes that a pentameric ATPase pushes the viral DNA by a “lever” into the procapsid in a fashion that the DNA rotates and rolls inside the motor channel during packaging process.

Recently, a fourth, the one-way revolution model, has been proposed by Guo, Schwartz, and co-workers (21–27), according to which the hexa-meric ATPase actively pushes the DNA into the procapsid, and the four electropositive lysine rings of the connector channel facilitate the DNA revolution, albeit without DNA rotation inside the channel during the packaging process. Simultaneously, the connector acts like a one-way valve and prevents leakage of the viral DNA against large counterpressure.

To reveal the molecular determinants of the exceptional mechanical stability and pressure resistance, we first probed the mechanical properties of the connector by equilibrium and nonequilibrium (force probe) molecular dynamics (MD) simulations (28–30). Remarkably, it turned out that the channel formed by the middle region of the connector is one of the stiffest known protein materials. To test which of the above packaging models is compatible with the properties of the connector, we next computed the deformation free energies required for twisting-untwisting as well as for compression-stretching motions both by a fluctuation

analysis of equilibrium MD simulations and by free energy umbrella sampling simulations. Our results demonstrate that the untwisting of the connector by 12°, as required by the untwist-twist model, would require much more energy than the ~50 kJ/mol provided by hydrolysis of one ATP molecule. Additionally, in recent voltage-ramping experiments (27), the connector facilitated DNA transport across the membrane even in the absence of the ATPase. These results are difficult to reconcile with this model, and thus render either the push-and-roll or the one-way revolution model more likely.

METHODS

Modeling and structure refinement

Four x-ray crystal structures of the connector at different resolutions have been published (9,15,31). We used the highest-resolution structure (PDB:1H5W; 2.10 Å) as a starting structure for all subsequent simulations. In none of the above structures, the residues ranging from A230 to S244 were resolved. These form a loop pointing toward the DNA and most likely interact with the DNA. (These were modeled and further refined as described in Section S1 in the *Supporting Material*, including Table S1, Fig. S1, and Fig. S2.)

Equilibrium MD simulations

The connector was extracted from the refined connector-DNA complex and taken as starting structure for subsequent equilibrium MD simulations. The simulation system was set up as follows: the connector was placed within a dodecahedron periodic box, solvated by addition of 91,559 water molecules, and neutralized by 84 sodium ions. The complete simulation system comprised 326,925 atoms. This system was energy-minimized and then heated up to 300 K during an NVT simulation with 1 fs time step. During this period, all heavy atoms were restrained with a harmonic force constant of 1000 kJ/(mol nm²). During a subsequent 3 ns NPT simulation, the restraints for the heavy atoms were gradually removed. Finally, a 200 ns free equilibrium MD simulation was performed using a 2-fs time step.

All simulations were carried out using the GROMACS 4.0.7 package (32) with the AMBER ff99SB force field (33) and TIP3P water model (34). Long-range electrostatic interactions were computed by the particle-mesh Ewald method with a grid spacing of 1.2 Å and fourth-order cubic interpolation (35). Short-range nonbonded interactions were computed for all atom pairs within a 10 Å cutoff. The temperature was maintained at 300 K using the V-rescale algorithm (36) with a coupling time constant of 0.1 ps; during the equilibration and free equilibrium MD simulations, the pressure was maintained at 1 atm using Berendsen and Parrinello-Rahman pressure couplings (37,38) with a 1 ps coupling time constant, respectively. All bonds were constrained using the parallel LINCS implementation (39,40). The VMD program was used for visualization (41).

Umbrella sampling simulations

Deformation free energy profiles for twisting-untwisting and compression-stretching motions of the connector were calculated from umbrella sampling simulations. To obtain the reaction coordinates for umbrella sampling simulations, we performed several force-probe (FP-1, FP-2, FP-3, FP-4, FP-5, and FP-6) and relaxation (Relax-1, Relax-2, Relax-3, and Relax-4) simulations (see Section 3 and Table S2 of the *Supporting Material* for more details.) For the compression-stretching motion, 14 sampling windows were prepared, with umbrella potential minima between $L_m = 2.725$ and 3.149 nm (for details, see Table S3). Appropriate starting struc-

tures for these simulations were taken from snapshots at various times from the Relax-1, Relax-2, Relax-3, Relax-4, and equilibrium simulations. Each umbrella sampling simulation was performed for 100 ns (total 14×100 ns), and otherwise identical to the equilibrium simulations described above. The first 50 ns of each umbrella sampling simulation were discarded as an equilibration phase; the last 50 ns were used for collecting histograms (see Fig. S4), from which deformation free energy was calculated using by weighted histogram analysis method (WHAM) (42–44).

Deformation free energy for the twisting-untwisting motion was obtained in a similar manner, using the twist angle θ_m as a reaction coordinate (see Fig. 1 E). Fourteen umbrella windows were defined for which starting structures were taken from the FP-5 and FP-6 simulations. Because the conformations were close to the minimum in the energy landscape during these simulations compared with other force probe simulations, we have selected snapshots at different times from these two simulations as starting structures (for details, see Table S4). The umbrella sampling simulations were preceded by an equilibration simulation for 25 ns to equilibrate the connector structure at each respective umbrella potential minimum (total 14×25 ns). In these simulations, the atoms of the reference and rotational group (*upper* and *lower disk* in Fig. 1 E, respectively) were harmonically restrained by a 10,000 kJ/(mol nm²) of force constant at the starting position such that the structures equilibrated at the starting twist angle of each umbrella window. Subsequently, two sets of umbrella sampling simulations were performed (15 ns for each window, total 28×15 ns). For the first set, the positions of reference atoms in the upper disk (shown in Fig. 1 E) were restrained while those of the bottom disk were subjected to an isotropic pivotal free rotational potential (29), and vice versa for the second set of simulations. The harmonic force constants used in simulations are listed in Table S4. To calculate the deformation free energy of the twisting-untwisting motion, a torsional spring constant for each window was calculated from the applied torque and resulting twist angle (for details, see Fig. S5). Histograms were collected from the umbrella sampling simulations and the deformation free energy was calculated using weighted histogram analysis method (WHAM) (42–44).

Analysis

Structural descriptors for the elastic properties

To probe the elastic properties of the connector, two structural descriptors, namely the twist angle and the length, were considered that describe the twisting-untwisting and compression-stretching motion of the connector, respectively. The connector length L is the distance between the center of masses of the upper and the bottom region (see Fig. 1 C). The whole connector's twist angle

$$\theta = \frac{1}{12} \sum_{i=1}^{12} \theta_i$$

has been calculated by averaging over the twist angle θ_i of each subunit (see in Fig. 1 C). Similarly, the twist angle θ_m and length L_m of the middle region were calculated to investigate the elasticity of the channel, where θ_m denotes the rotational angle between the upper and the lower disk and L_m the distance between the two disks shown in Fig. 1 D. The elastic properties were calculated using the snapshots taken at 2 ps time difference during the last 100 ns of equilibrium MD simulations.

Elastic properties and spring constants from equilibrium simulations

The free energy landscape $G(\theta, L)$ as a function of the connector length L and angle θ as defined above was determined independently from equilibrium simulations in harmonic approximation from the equilibrium fluctuations of the connector via

$$G(\theta, L) = -k_B T \ln p(\theta, L),$$

where k_B and T are the Boltzmann constant and temperature, respectively. For a Gaussian approximation of the two degrees of freedom, θ and L , probability density is given as

$$p(\theta, L) \propto \exp\left(-\frac{1}{2} [(\theta - \bar{\theta}), (L - \bar{L})] \mathbf{C}^{-1} \begin{bmatrix} (\theta - \bar{\theta}) \\ (L - \bar{L}) \end{bmatrix}\right), \quad (1)$$

where $\bar{\theta}$ and \bar{L} are the average twist angle and length, respectively, and \mathbf{C} is the covariance matrix. Because the energy landscape is assumed to be harmonic, its expression is

$$G(\theta, L) = \frac{1}{2} \left[K_\theta (\theta - \bar{\theta})^2 + K_L (L - \bar{L})^2 \right] + K_c (\theta - \bar{\theta})(L - \bar{L}),$$

where K_θ is the torsional spring constant, K_L is the stretching spring constant, and K_c is the coupling constant between two motions. By comparing the above three equations, these constants can be written as

$$\begin{bmatrix} K_\theta & K_c \\ K_c & K_L \end{bmatrix} = k_B T \mathbf{C}^{-1}. \quad (2)$$

This equation was used to calculate the spring constants from the equilibrium fluctuation for the whole connector and the middle region-containing channel.

Young's modulus of elasticity

To translate the obtained elastic constant into a Young's modulus of the connector, we described the connector as a homogeneous elastic material with elasticity Y and with the shape of a hollow truncated cone of length L (see Fig. S6). From the observed length change of

$$\delta = \int_0^L \frac{F}{YA(x)} dx$$

upon axial stress force F (45), where $A(x)$ is the cross-sectional area perpendicular to the symmetry axis, the elasticity Y in terms of stretching spring constant K_L is

$$Y = \int_0^L \frac{K_L}{A(x)} dx, \quad (3)$$

which yields, for the assumed hollow truncated cone having variant diameters,

$$Y = \frac{2K_L}{\pi} \frac{L}{D_b d_a - D_a d_b} \ln \left| \frac{(D_a + d_a)(D_b - d_b)}{(D_a - d_a)(D_b + d_b)} \right|, \quad (4)$$

where D_a and d_a are narrow-end exterior and interior diameter, respectively, and D_b and d_b are wide-end exterior and interior diameter of the truncated cone, respectively (*sketch* shown in Fig. S6). The dimensions are calculated for the whole connector and the middle region from equilibrium MD simulations and provided in Table S5.

Calculation of interface area

To quantify inter- and intrasubunit residue packing, we have calculated surface area (SA) accessible by a probe with a radius of 1 Å using the *g_sas* module of the GROMACS package (46). Intrasubunit packing was determined via helices MH1, MH2, and MH3 of the middle region (Fig. 1 C),

$$\begin{aligned} SA^{\text{intra}} = & [(SA_{MH2} + SA_{MH3}) - SA_{MH2-MH3}] / 2 \\ & + [(SA_{MH2-MH3} + SA_{MH1}) - SA_{MH1-MH2-MH3}] / 2, \end{aligned} \quad (5)$$

where subscripts denote either separate helix or attached pair helices. Similarly, intersubunit packing was calculated via

$$SA^{\text{inter}} = [(SA_i + SA_j) - (SA_{ij})] / 2, \quad (6)$$

where SA_i and SA_j refer to separated adjacent subunits, and SA_{ij} to the attached subunits.

RESULTS AND DISCUSSION

We first monitored the structural stability of the connector during equilibrium MD simulations via root mean-square deviation calculated for all C-α atoms with respect to the crystal structure (see Section S2, Fig. S7, and Fig. S8 in the Supporting Material). Overall, the structural changes during 200-ns equilibration were rather small, with the root mean-square deviation stabilizing at 0.25 nm. The last 100-ns part of the trajectory was used for further analysis.

How does the connector withstand the high pressure difference?

Elasticity of the connector from equilibrium fluctuations

To study the structural determinants that enable the connector and its channel to withstand large relative pressure, we first calculated the elastic properties of the whole connector as well as its middle region (see Table S6). The stiffness opposing twisting-untwisting and compression-stretching motions (see Fig. 1, C and D), described by torsional (K_θ) and stretching (K_L) spring constants (see Table S6) along with coupling constants (K_c) between motions, was determined using Eq. 2 from the equilibrium fluctuations shown in Fig. 2, A and B. As can be seen, the calculated torsional and stretching spring constants of the middle region are approximately two and eight times larger than that of the whole connector, respectively; therefore, most structural changes under pressure are expected for the upper and bottom regions.

Using the stretching spring constants and taking into account the geometry of the connector (see Eq. 4), Young's moduli of 0.36 ± 0.06 GPa and 3.4 ± 0.6 GPa for the whole connector and for the middle region, respectively, were obtained. Furthermore, the obtained moduli values were almost unchanged during the last 25 ns of the simulations that suggest these values are sufficiently converged (see Fig. S9). A similar convergence behavior has been seen also for a similar-sized molecular system, i.e., the central shaft γ-subunit of the F1-ATPase motor, for which, additionally, good agreement with experimental results has been shown (47).

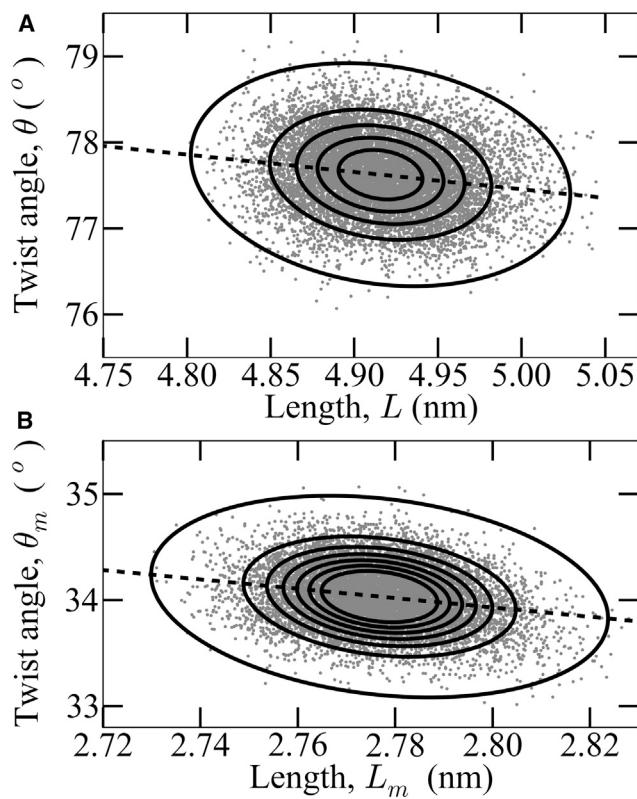


FIGURE 2 Equilibrium fluctuations of twist angles and lengths. (A) Fluctuations of twist angle θ and length L of the whole connector, derived from equilibrium simulations (shaded dots). The probability density was obtained using Eq. 1 (depicted as solid lines ($2^{\circ} \text{ nm}^{-1}$ spacing)). (B) Same representation for twist angle θ_m and length L_m and $5^{\circ} \text{ nm}^{-1}$ contour line spacing. Linear least-square fits (dashed lines) quantify the coupling between compression-stretching and twisting-untwisting motions.

For the whole ϕ 29 procapsid, a Young's modulus of ~ 1.8 GPa was measured in AFM experiments (16,17); thus the connector as a whole appears to be softer than the procapsid, with a stiffer "core" inside. The elasticity of this middle region is similar to that of other structural proteins that withstand mechanical forces, such as collagen fibrils (0.2–11.5 GPa) (48), single-brin silkworm silk (5–17 GPa) (49), or dragline spider silk (11–13 GPa) (50). We now ask what are the structural determinants and the function of this heterogeneity.

Heterogeneous flexibility of the connector

To characterize the spatial distribution of the observed elastic heterogeneity as well as its relation to molecular fluctuations, we calculated, residuewise, root mean-square fluctuations (RMSF) for each subunit. Fig. 3 A (green line) depicts the obtained values averaged over the 12 subunits. As can be seen, overall the crystallographic temperature factors (blue lines, taken from Guasch et al. (15)) agree well with the RMSF (correlation coefficient: 0.83), which suggests that these fluctuations and the derived elastic

properties are captured sufficiently accurately by our simulations.

To characterize the spatial distribution of the elastic properties, we identified rigid regions from RMSF depicted in Fig. 3 A. Residues with small fluctuations (RMSF < 0.065 nm) were considered rigid (red region in Fig. 3, B–D), whereas those residues with larger fluctuations were considered flexible (blue region). In the upper region, rigid residues are embedded within flexible parts, very much like in composite materials. The middle region is the stiffest part and mainly consists of rigid residues. In contrast, the residues of the hinge and the bottom regions are flexible (Fig. 3 B). This finding suggests that the relatively low stiffness probed by AFM (16) is mainly due to the flexible and elastic bottom region of the connector.

Obviously, the regions defined in Fig. 1 B purely based on structural features also reflect different elastic properties of the connector. This mechanic heterogeneity suggests that these regions even fulfill different functions. As visible from Fig. 3 C, the pressure acts primarily on the rigid core (red), which defines the channel that is in direct contact to the DNA. To maintain a constant diameter of the channel, this region, therefore, has to withstand deformations due to the strong pressure gradient between the capsid interior and exterior as well as mechanical stress via the contacting parts of the procapsid, both in the presence and absence of DNA. Due to the functional relevance of this region, one would expect the structure of this region to be highly conserved. Indeed, very similar rigid helical scaffolds are also seen in other tailed phage connectors such as T7, SPP1, and P22 (51–57). Rigid residues are located inside the procapsid whereas the flexible bottom region is located outside of the procapsid.

Interestingly, the rigid core is directly exposed to the solvent of the capsid interior (arrows pointing high pressure in Fig. 3 C); here, the exposure to solvent evenly distributes the pressure over the surface of the core region. In contrast, contact of the upper region to the procapsid is buffered by flexible regions, which absorb local force peaks and distribute external forces evenly over the outer surface of the core region. In that respect, and also from the compartmented substructure of the outer rim visible in Fig. 3 B, this region resembles a composite material, very similar to the protein nanocrystals that are embedded within flexible unstructured peptide regions in silk (58) or the calcium carbonate (calcite or aragonite) crystals layers within a protein matrix in sea shells (59,60). From closer analysis of the direct contact region to the capsid (sketched in Fig. 3 D) we speculate that this structure particularly serves to withstand displacement of the connector due to vertical forces from the capsid interior, while tolerating horizontal motions or forces that the capsid channel rim may exert onto the middle region of the connector.

The flexible bottom region points toward the capsid exterior and, therefore, does not need to withstand large pressure

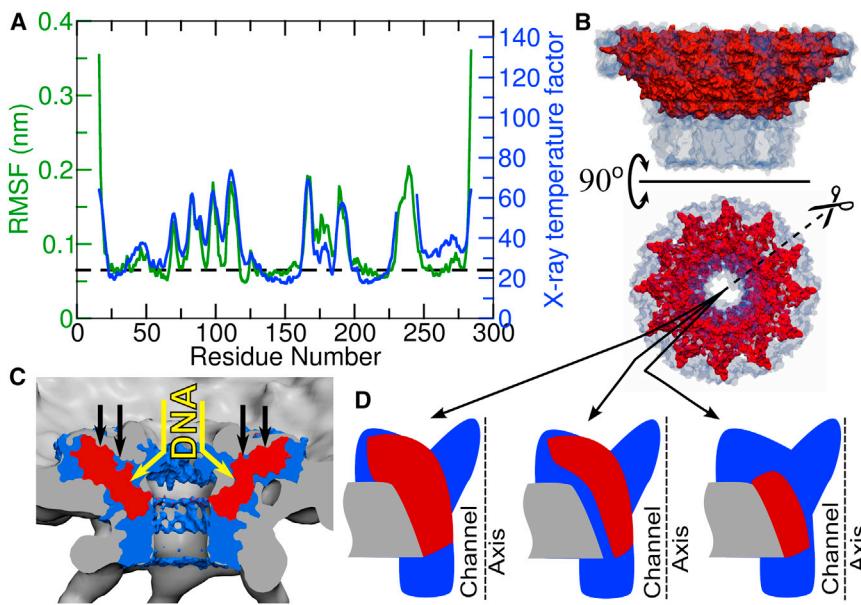


FIGURE 3 Heterogeneous distribution of rigid and flexible connector regions. (A) Root mean-square fluctuations (RMSF) (green) and temperature factors (blue) obtained from crystallography (15). Residues with an RMSF below 0.065 nm (horizontal dashed line) are considered rigid, otherwise flexible. (B) Spatial distribution of rigid (red) and flexible (blue) connector regions (side and top view) and (C) shown embedded within the procapsid (gray). Mainly rigid residues of the middle region (red) are exposed to pressure (black arrows) due to compacted DNA. This rigid region also supports loops formed by residues A230–S244 that directly interact with the DNA (yellow arrows). (D) Three different types of the connector-procapsid interfaces are illustrated by three schematic radial cross-sections (indicated by three arrows) of the connector (flexible regions in blue, rigid regions in red) and the part of the procapsid (gray) that is in direct contact with the connector. An alternating pattern of direct contacts is seen between the central rigid region (red), the rim of the procapsid (gray), and regions where flexible regions (blue) act as an interface.

differences or mechanical stress. Accordingly, its high flexibility might facilitate binding of the pRNA and ATPase at the start of the packaging process. Overall, the heterogeneous flexibility pattern seen in our simulations of the ϕ 29 connector closely matches the mechanical properties that are required to perform its biological function within the extreme mechanical and pressure conditions of its procapsid environment.

Elastic properties determined from force probe simulations

Above, the elastic properties of the connector were probed via equilibrium simulations. Accordingly, the probed length and twist angle changes only covered the range accessible to thermal fluctuations. Further, by calculating elastic constants, we assumed a harmonic (i.e., Hookean) and reversible behavior of the connector. However, the different models of the packaging mechanism, as discussed in the Introduction, require much larger connector deformations than those observed above; it is thus unclear if the connector also exhibits Hookean properties in this case, which also would require a fully elastic, i.e., reversible behavior. We therefore need to determine its elastic limit, i.e., to which extent it can be deformed reversibly. It is, finally, unclear if the potentially functionally relevant coupling between stretching and twisting of the connector, as calculated from the equilibrium fluctuations shown in Fig. 2 A, also extends into deformations that are thermally inaccessible.

To address these questions, we carried out several sets of force probe (FP) simulations, which are presented and discussed in Section S3 of the Supporting Material (see Table S2, Fig. S10, Fig. S11, Movie S1, and Movie S2 in the Supporting Material). In summary, deformations within the range of $L = 4.70\text{--}5.3$ nm and $\theta = 70\text{--}79.5^\circ$ seem to be fully elastic, and the twisting-untwisting motion is linearly

coupled to the compression-stretching motion within this elastic range. The connector recovered its equilibrium structure even after structural breakdown within the obtained elastic limit (see Fig. S12 and Fig. S13). The obtained coupling of $18^\circ/\text{nm}$ is remarkably close to the coupling of $17.6^\circ/\text{nm}$ required in the untwist-twist DNA packaging model proposed by Simpson et al. (9). We therefore asked next if the other elastic and energetic properties of the connector are also compatible with this model.

Role of the connector in the DNA packaging process

Twisting-untwisting of the connector

Specifically, according to the untwist-twist model, the coupled untwisting and stretching motion of the connector by 12° and 0.68 nm, respectively, is driven by hydrolysis of one ATP molecule, which under physiological conditions releases an energy of ~ 50 kJ/mol.

As a first step, to check if this energy suffices to induce the above deformation, we estimated the required free energy in harmonic approximation from the equilibrium fluctuations (see gray dots in Fig. S14), as described in the Methods. Extrapolation of this harmonic approximation (depicted as contour lines) suggests, however, that a very large amount is actually required for the deformation of $12^\circ/0.68$ nm, >10 times larger than the 50 kJ/mol available from ATP hydrolysis.

However, the above force-probe simulations (see Fig. S10, B and C) suggest that the simple harmonic approximation may not hold for such extreme deformations.

In a second step, we therefore computed the potential of mean force (Fig. 4, A and B) along both the

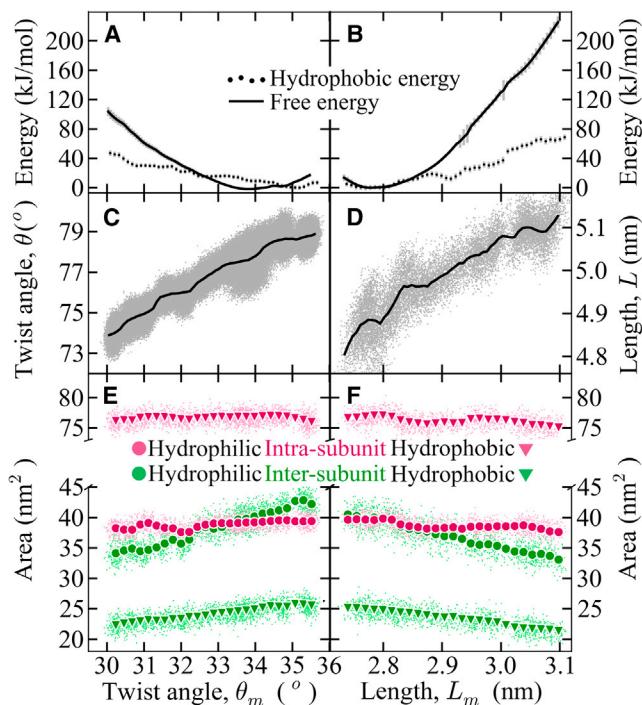


FIGURE 4 Deformation free energies and exposure of hydrophobic and hydrophilic residues of the connector from umbrella sampling simulations. Compressing-stretching deformations (L , L_m) and twisting-untwisting deformations (θ and θ_m) for the whole connector and, respectively, the middle region, are shown. (A and B) Deformation free energy (black solid line) as well as the contributions of hydrophobic solvent-accessible surface area to the free energies (black dotted lines) for (A) twisting-untwisting and (B) compression-stretching deformation of the connector middle region. (C and D) Coupling between deformations of the whole connector and respective middle region deformations (small gray dots), with running averages (solid lines). (E and F) Change of intersubunit (green) and intra-subunit (red) interface contact areas (small dots, as defined in the text) during (E) twisting-untwisting and (F) compression-stretching deformations of the middle region; decreasing hydrophobic (triangle) and hydrophilic (circle) contact areas imply increasing solvent exposure. Running averages are shown as larger symbols.

twisting-untwisting (θ_m) as well as the compression-stretching (L_m) coordinate from umbrella sampling simulations using the weighted histogram analysis method (see Methods for details). As can be seen from the first set of umbrella sampling simulations with length (L_m) of the middle region of the connector as reaction coordinate (Fig. 4, B and D), the available 50 kJ/mol allow for an extension of only 0.1 nm, thus confirming the above extrapolation. Similarly, using the twist angle (θ_m) as a reaction coordinate, already ~100 kJ/mol are required to untwist both the middle region and the whole connector by only 4° (Fig. 4, A and C), i.e., by one-third of the deformation required by the proposed untwist-twist model. Therefore, our umbrella calculations also strongly suggest that the free energy released by ATP hydrolysis is too small for the required deformations.

Furthermore, to characterize the coupling between the deformations of the middle region and the whole connector

that was proposed in the untwist-twist model, we determined the whole connector's deformation, i.e., the change in the twist angle (θ) and the length (L) with respect to that (θ_m and L_m) of the middle region (Fig. 4, C and D). As can be seen, the deformation of middle region entirely translates to the whole connector, which suggests that the deformations of the whole connector and the middle region are indeed strongly coupled.

We note that our above estimate of the deformation free energy rests on the assumption that it does not decrease with stretching or untwisting beyond $L_m = 3.1$ nm and $\theta_m = 30^\circ$, as might happen, e.g., after complete structural breakdown of the connector. However, the reversible recovery of the equilibrium conformation after deformations of up to 5.1 and 5.3 nm, respectively (see Fig. S13, A and B), strongly speaks against a complete structural breakdown.

To assess the convergence of the umbrella sampling simulations, we compared the resulting untwisting-stretching path from the first set of simulations with the second set (shown in Fig. S15). These two paths are similar to each other (red and black symbols) and also coincide with the path obtained in FP-5/6 simulations (green symbols), underscoring that the free energy profiles are nearly converged.

All potentials of mean force were calculated in absence of the DNA, which may affect the energy required for the proposed untwisting/stretching. However, according to the above results, already half of the proposed deformation requires ~200 kJ/mol. In contrast, the maximum barrier height that can be overcome with the available 50 kJ/mol for an activated process is ~100 kJ/mol, assuming an additional barrier of 50 kJ/mol, estimated from a Kramer's attempt rate of $10^{10}/\text{s}$ and a plausible turnover rate of 165/s (14). We consider it therefore unlikely that the presence of DNA would decrease the required deformation free energy by such a large amount to drive the deformations required by the untwist-twist model. Interestingly, Jing et al. (27) observed in their voltage-ramping experiments that DNA was driven by the electrochemical gradient through the connector even in the absence of pRNA and ATPase. According to the Nernst equation, the applied potential of 75 mV (= 7.24 kJ/mol) translates, for the two basepairs (eight negative charges) by which the DNA is advanced in one cycle, to an energy of ~58 kJ/mol. In these experiments, this is thus the maximal available energy for possible connector deformations—again much smaller than required by the untwist-twist model. Combined with our above results, the observed DNA diffusion in the absence of an ATPase strongly suggests that in these experiments, the connector also facilitates DNA transport without performing the proposed twisting-untwisting or compression-stretching motion.

The connector as a one-way valve

As alternatives, the so-called push-roll and one-way revolution models have recently been proposed (12,21). Both

models postulate that the ATPase interacts with and actively transports the viral DNA. Additionally, the latter model proposes that the connector acts as a one-way valve, restricting leakage of the DNA during the packaging process (21). In fact, in the above-mentioned voltage-ramping experiments, selective one-directional diffusion of DNA across the lipid membrane through the connector was observed; for a reversed potential, no backdiffusion of the DNA was seen (27). Further, the connector's ability to restrict the leakage of DNA was probed through sedimentation assays in which the DNA was retained inside partially filled procapsids in the absence of pRNA/ATPase after ultracentrifugation (27).

Furthermore, in mutagenesis experiments, deletion/mutation of particular connector loop residues (K234–R237; loops depicted in Fig. S1 B and Fig. S2 C) severely affected the successful production of infectious bacteriophage (61). In sedimentation assays, the mutant K235A.E236A.R237A was unable to retain DNA inside completely filled procapsids in the presence of pRNA/ATPase. These results suggested that the connector loops are essential for the observed unidirectional DNA transport (26) and strongly interact with the DNA via three positively charged residues (K234, K235, and R237) per each subunit (61). One possible mechanism that would involve the connector loops as a key is that the connector acts as a check-valve, with the flexible loops bound to the DNA acting as movable parts that obstruct the channel and arrest the DNA against backmotion toward the outside, due to the strong pressure force. Such mechanism, to function, would require a rigid anchoring of the loops to withstand the backtracking force acting onto the DNA. Indeed, this particular loop region is solidly attached to and supported by the stiff region of the connector that has been identified above (*red region*, Fig. 3, B and C). Further, recent voltage-ramping experiments revealed that after deletion of these loops, the connector failed to restrict the backtransport of the DNA across the lipid membrane (21). Overall, the obtained heterogeneity in the mechanical properties of the connector seems to be compatible with the one-way revolution model.

Structure and energetic determinants for connector stiffness

To answer the question of which features of the connector determine its remarkable mechanical properties, we calculated and analyzed the extent to which selected structural and energetic quantities changed during the deformations induced by the above-described umbrella sampling simulations. As one possible source of the observed elastic restoring forces, we focused at the hydrophobic core (62–64) of the middle region of the connector. Should this core become partly exposed to the solvent during untwisting or stretching motions, the corresponding free energy cost might explain these forces. To test if this is actually the case, we calculated the hydrophobic solvent-accessible sur-

face areas of the middle region (see Fig. S16) and estimated the resulting hydrophobic free energy from an energy cost of 18 cal/mol per unit surface area (\AA^{-2}) (65) (*dotted lines* in Fig. 4, A and B). As can be seen, the hydrophobic energy indeed contributes approximately one-third to the total free energy, but clearly does not fully explain the connector's elastic properties.

We then asked if a possible loosening of the packing within the connector, and the resulting loss of intra- and intersubunit interactions within the middle regions, might also contribute to the mechanical properties. To this aim, we have quantified the packing within each subunit by the intrasubunit packing (Eq. 5), defined by the interface area (Fig. 4, E and F, *red symbols*) between each of the three helices (MH1, MH2, and MH3 shown in Fig. 1 C), as well as the packing between adjacent subunits (Eq. 6), defined by the interface area between two adjacent subunits (*green symbols*). Further, Fig. 4, E and F, distinguishes between hydrophobic and hydrophilic interface surface.

For the intrasubunit packing, the hydrophobic area was approximately two times larger than the hydrophilic area, suggesting the presence of an internal hydrophobic “core” within each subunit. However, no significant changes of the respective areas are present during both untwisting and stretching, such that their contribution to the deformation free energy is small. In contrast, both intersubunit hydrophilic and hydrophobic interface areas decreased during stretching and untwisting, indicating a loosening of their mutual packing. That this loosening actually implies significant interaction enthalpy changes is demonstrated in Fig. S17, which shows the respective van der Waals and electrostatic energies during deformation. Because, due to the size of the simulation system, the corresponding entropic part could not be quantified, we cannot decide whether this second free energy contribution, together with the hydrophobic surface changes quantified above, suffices to fully explain the deformation free energies derived from our umbrella sampling simulations. However, we consider it unlikely that the respective enthalpy/entropy reduces the resulting free energy to such a large extent as to render the intersubunit packing changes unimportant.

CONCLUSIONS

During assembly of many bacteriophages, strong motors transport the viral DNA inside the phage procapsid through a connector against pressure difference of up to ~60 atm. Here we have investigated the mechanical elastic properties of the ϕ 29 head-tail connector (a component of the ϕ 29 bacteriophage DNA packaging motor), and its possible role in the DNA packaging mechanism. A fluctuation analysis of equilibrium MD simulations revealed an exceptionally stiff α -helical channel region, with an elastic modulus that is only approached by proteins that have evolved to withstand strong mechanical forces, such as crystalline

silk domains. The functional relevance of this middle region is underscored by its high conservation level in head-tail connectors of other phages such as SPP1 and P22 (52,54–56). The high stiffness may serve to stabilize the channel and in particular to maintain its inner diameter in absence of DNA. Remarkably, soft regions were also seen within the connector, such that, overall, the connector displays an unexpected mechanical heterogeneity, resembling that of typical composite materials such as silk and sea shells—not only structurally but also, most likely, functionally.

Our force probe simulations revealed a large elastic regime of the connector, and showed that quite large untwisting-twisting and compression-stretching deformations are in fact reversible on very short timescales. The extent of fully reversible deformations is markedly larger than that typically seen for proteins such as immunoglobulin domains or ubiquitin (66,67), but comparable to proteins with functionally important elasticity such as importin- β (68,69) or viral capsid proteins (30,70). It will be interesting to study how mechanical properties of the connector might change in the presence of DNA.

Subsequent closer analysis of umbrella sampling trajectories revealed that the observed pronounced exposure of hydrophobic residues to solvent during deformation markedly contributes to the total free energy required to deform the connector, as also been observed for viral capsid proteins (70). Upon deformation of the connector, its hydrophobic residues core is exposed to the solvent, particularly at the intersubunit interface, which suggests that this core is a structural feature that contributes to the exceptional stiffness of the connector. Further, the interaction enthalpies between adjacent subunits were observed to weaken during the deformations, which suggests an additional, mainly enthalpic, contribution to the overall elasticity.

Our results also shine light on the four possible different packaging mechanisms that have been proposed (9,12,13,18–20,26,27,61). In particular, the free energy required to deform the connector to an extent required for the untwist-twist mechanism is much larger than the energy provided by ATP hydrolysis, which—combined with results from voltage-ramping experiments (27)—speak against that mechanism. Our results neither rule out nor support the first proposed mechanism, involving full rotations of the connector (18); however, this mechanism has already been ruled out experimentally (19).

The heterogeneous stiffness pattern of the connector and its elastic properties, as obtained from our simulations, are compatible with the fourth, recently proposed one-way revolution mechanism. According to this mechanism, the connector acts as a one-way valve and thereby restricts leakage of the viral DNA during and after the DNA packaging process. In particular, strong interactions are expected between flexible loops located at the inner rim of the channel formed by residues K234–R237, which can obstruct the

channel similar to a check-valve mechanism, whereas the stiff regions of the connector provide a solid anchor for these flexible loops. We note that, because no structural information is available on the bound DNA, it has not been included in our simulations and, therefore, our results neither rule out nor support the DNA revolution mechanism that is proposed in the one-way revolution model. Similarly, our results neither rule out nor support the DNA rotation-roll mechanism that is proposed in the third proposed model, the push-roll model. To that aim, it remains to be tested how the connector interacts with the pRNA and ATPase to facilitate DNA translocation and how the viral DNA moves into the procapsid during the packaging process.

SUPPORTING MATERIAL

Six tables, 17 figures, two movies and References (71–80) are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(14\)00126-X](http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00126-X).

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