Switch interactions control energy frustration and multiple flagellar filament structures

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Bacterial flagellar filament is a macromolecular assembly consisting of a single protein, flagellin. Bacterial swimming is controlled by the conformational transitions of this filament between left- and right-handed supercoils induced by the flagellar motor torque. We present a massive molecular dynamics simulation that was successful in constructing the atomic-level supercoil structures consistent with various experimental data and further in elucidating the detailed underlying molecular mechanisms of the polymorphic supercoiling. We have found that the following three types of interactions are keys to understanding the supercoiling mechanism. "Permanent" interactions are always maintained between subunits in the various supercoil structures. "Sliding" interactions are formed between variable hydrophilic or hydrophobic residue pairs, allowing intersubunit shear without large change in energy. The formation and breakage of "switch" interactions stabilize inter- and intrasubunit interactions, respectively. We conclude that polymorphic supercoiling is due to the energy frustration between them. The transition between supercoils is achieved by a "transform and relax" mechanism: the filament structure is geometrically transformed rapidly and then slowly relaxes to energetically metastable states by rearranging interactions.

bacterial swimming | molecular dynamics | supercoiling | flagellin | transform and relax mechanism

The bacterial flagellum is a biological nanomachine for the locomotion of bacteria. The flagellum comprises three parts: the basal body as a rotary motor, the filament as a helical propeller, and the hook as a universal joint that connects the motor with the filament. When the motor rotates in a counterclockwise direction, several flagellar filaments of a left-handed helical structure form a bundle and act as a screw to move the bacteria straight (run). When the motor reverses its rotation, a transition of the filament structure into a right-handed helix is induced, the bundle is untangled, and bacteria change the direction of movement (tumble) (1). The flagellar filament is a tubular supercoil structure consisting of subunits composed of a single protein, flagellin. This structure can be described as stacked helical units, each consisting of 11 subunits (elementary helical step hereinafter) of a single protein, flagellin. This structure can be described as stacked helical units, each consisting of 11 subunits, or as four circularly arranged protofilaments each forming nearly longitudinal helical arrays of subunits (2). The filament can be transformed into various distinct supercoil forms by changes in chemical environment (3–5), single amino acid mutations (6, 7), or mechanical forces (8, 9). The polymorphic state in the "run" mode is called "normal," and in the "tumble" mode the state is either in "semicoil," "curly I," or "curly II" (8, 10). From a static point of view, the polymorphism of supercoils is reasonably well understood as a bistable protofilament model (11–14) in which subunit conformation is assumed to be the same within each protofilament, and protofilament conformation including the interactions with neighboring protofilaments is classified into two distinct forms: R-type and L-type. Two aspects, the assumed intrinsic bistable character of the protofilament and geometrical constraints coming from the formation of a tubular structure, result in discrete polymorphic states of the flagellar filament, as characterized by combination of the R- and L-type protofilaments, i.e., normal (2R/9L), semicoil (4R/7L), curly I (5R/6L), and curly II (6R/5L) (15, 16). Although the bistable protofilament model gives a reasonable static and mechanical view of polymorphic supercoiling on the nanoscale, the underlying molecular mechanisms are not yet fully understood.

Here, we present a previously undescribed polymorphic supercoiling mechanism, which was deduced from massive 2.4-megamolecule simulations (MD) simulations for 20 ns. In contrast to the semimicroscopic mechanical bistable protofilament model, we give a microscopic view in which an essential role is played by thermal fluctuations, and these fluctuations in turn are responsible for softness of protein structures (17–19). From the microscopic view we also present a molecular mechanism for transitions between different supercoiled states.

Results and Discussion

Stability of R-Type Straight Filament Models. The A449V flagellin mutant from SJJW1655 strain of Salmonella typhimurium forms a straight filament, whose protofilaments are all in R-type. Its atomic structure recently was solved by x-ray crystallography for the F41 fragment of flagellin (41 kDa) (20) and by electron cryomicroscopy for the whole filament (21). The simulated system, whose initial atomic coordinates were taken from the latter, contains a short filament consisting of 44 flagellin subunits, i.e., four helical units made of 316,668 atoms (Fig. 1d) and surrounding water molecules and counterions (see Materials and Methods). The R-type straight filaments were also constructed with wild-type (WT) (SJJW1103) and G426A mutant (SJJW1160) flagellin, which are known to form "normal (2R/9L)" and L-type straight (0R/11L) filaments in the native state, respectively. The helical form of the filaments can be characterized by two parameters, twist and curvature. For each instantaneous structure (snapshot), the local twist and curvature between the two central 11 subunits (elementary helical step hereinafter) were measured (see Materials and Methods). The structure of the A449V filament, which is expected to take the R-type straight form, is actually confined within the vicinity of the initial straight structure during the simulation, while the WT and G426A filaments drifted away from it (see Fig. 5, which is published as supplementary material on the PNAS web site).

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Abbreviation: MD, molecular dynamics.

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supporting information on the PNAS web site). This computational observation of stability and instability in the straight form in the first and latter two cases, respectively, does agree with the expectation from experiments.

**Finding Polymorphic Supercoil Structures.** Starting from the structure obtained above, a torque was applied by force–bias MD to the filament model composed of WT flagellin to find whether polymorphic supercoil structures exist as energetically metastable states (see Materials and Methods). Long filaments consisting of 10,680 subunits are also shown (see Materials and Methods). (a) Initial structure for simulation. (b and c) Snapshots of normal (b) and semicoil (c) during simulation. (d) Helical parameters of metastable structures of 10 distinct MD trajectories (last 150 ps). Red “+” connected by lines indicates parameters expected from the bistable protofilament model. Long filaments consisting of 10,680 subunits are also shown (see Materials and Methods). (e) Structures of randomly generated long filaments. (f) Results of force–bias MD starting from right- toward left-handed supercoil. Magenta (Movie 1) and yellow (Movie 2) lines represent results obtained for a torque of 5.0 and 2.0 × 10^2 pN nm, respectively. Density map shows probability determined from 11 trajectories that reached near 2 R/H20862 9 L or 1 R/H20862 10 L. White circle, initial structure; black circle, final structures; black line, probable position of energy barrier; white arrow, expected relaxation pathway. (g) Same as f but in the opposite direction. The magenta (Movie 3) and yellow (Movie 4) lines represent the results for a torque of 4.0 and 2.0 × 10^2 pN nm, respectively. Density map was determined from 34 trajectories that reached near 4 R/H20862 7 L or 5 R/H20862 6 L. White triangle and square, transient structures experimentally observed (9). Figs. 1 a–c, 2 b, and 3 were created with RASMOL (37).
observed experimentally (10). Observation of these discrete polymorphic states in our simulation indicates that our very short filament model captures the essence of the flagellar filament embodied in the bistable protofilament model. Our filament model even correctly reproduced the absence of experimentally unobservable states (1R/10L and 3R/8L). This finding indicates that our model more faithfully captures the energy profile of the system than the bistable protofilament model.

Distribution of the data shown in Fig. 1d is expected to be larger than those in real system because even a small fluctuation in the elementary step is accumulated and amplified in the long filament. To estimate more realistic fluctuation of the twist and curvature, we assumed that microscopic-state variations along the flagellar filament can be replaced by temporal-state variation of the same elementary helical step observed during the simulation. Models of polymorphic flagellar supercoil structures were generated by repeatedly stacking elementary helical steps belonging to the same cluster in the twist–curvature space (see Materials and Methods). The twist and curvature of the flagellar filament thus generated were measured and are plotted in Fig. 1e. These data agreed remarkably well with the experimental results. Comparison of Fig. 1d and e showed that, when a tubule is formed from the ensemble of elementary helical steps, the degree of shape variations decreases drastically. This result indicates a “semimacroscopic” character of the tubular filament structure composed of “microscopic” helical steps.

**Key Interactions in Supercoiling.** After successfully reproducing various experimental results, we should then ask whether our simulation endorses the bistable protofilament model, and, if yes, determine the basis of the differences between the R- and L-type forms with regard to structure and interaction. In the flagellar filament, a reference subunit numbered 0 makes contact with eight surrounding subunits labeled +5, +6, +11, and +16 (Fig. 2a). A helical line along subunits −5, 0, and +5 is called 5-start, for example. In this work, we treat the flagellar filament as stacked helical units. The helical unit consisting of 11 subunits is in fact made of two turns of a 1-start helix (see figure 2 of ref. 16). Intersubunit interactions along 5-, 6-, and 16-starts involve those between neighboring protofilaments, whereas interactions along 11-start involve those within each protofilament. Domains D2 and D3 protrude into solvent and have almost no contribution to intersubunit contacts. Domains D0 and D1 make intersubunit contacts and compose the filament core. The level of variation in subunit conformation among different protofilaments is indicated by different colors in Fig. 2b. When locally best-fitted, the upper part of domain D1 was found to be significantly rigid compared with the other regions. Similar tendencies also were seen in the thermal fluctuation of each subunit during the simulations. In addition, the upper part of D1 also was found to be rigid in a 1-ns MD simulation of isolated F41 monomer in solution (results not shown). Therefore, it should be understood that this rigidity originates mainly from the intrinsic nature of the flagellin structure rather than from intersubunit interactions. Although we attempted various classifications, we could not classify the subunits as R- or L-type from a structural view only because the subunit structures show significant variations and fluctuations (for example, see Fig. 6, which is published as supporting information on the PNAS web site).

With regard to interactions, we have performed a systematic analysis of all types of interactions that may be correlated with different filament supercoiled states. No intrasubunit interactions were found to be correlated. Intersubunit interactions are classified into three types: “permanent” (same interacting pair throughout the simulation), “sliding” (same type of interactions, i.e., hydrophilic or hydrophobic, with variable partner), and “others.” Among the others category, those correlated with
We have now concluded that the molecular mechanisms behind the bistable character of the protofilament are “on–off” switch interactions between neighboring subunits. Therefore, we can now extend the concept of bistability from the “semimacroscopic” one, as defined for each neighboring subunit pair, i.e., R-type for absent switch interactions and L-type for others. As seen in Fig. 4, R- and L-type states microscopically defined for each subunit pair show significant fluctuations behind the bistability semimacroscopically defined by the bistable protofilament model. It should be noted that, although each subunit structure fluctuates significantly, the typical or average L-type subunit structure was slightly more extended along the protofilament than that of R-type (see Fig. 7), which is published as supporting information on the PNAS web site, as expected from experimental data (15, 16).

**Superciling Mechanism.** The presence of permanent, sliding, and switch interactions suggests the following supercoiling mechanism. Permanent interactions determine the overall architecture of the flagellar filament. In each of the interacting residue pairs, one residue is located in the relatively rigid region in the upper part of domain D1, and the other is located in the more flexible regions, either in the lower part of domain D1 or the “spoke” between domains D1 and D0. This arrangement assures flexibility in intersubunit distances without changing interacting pairs. Permanent interactions are mainly responsible for the geometrical constraints of the tubular structure. Sliding interactions provide a mechanism that allows large flexibility in the intersubunit interface without a large change in interaction energy. A similar mechanism was suggested for the protofilament of the flagellar hook to allow a large variation in the intersubunit distance while maintaining intersubunit contacts to achieve bending flexibility (24). The S101–T144 and T102–N132 always made intersubunit hydrophobic interactions with A45 of subunit +16. Interestingly, it has been shown experimentally that when N-terminal regions including D42 and D43 were deleted, filaments were severely destabilized (22).

**Mechanism of Polyomorphic Transition.** Conformational transitions of the filament model from a right- to left-handed supercoil and vice versa were driven by the torque applied on domains D0 and D1 to simulate the torque produced by the rotary motor (see Materials and Methods) as shown in Fig. 1 f and g and also in Movies 1–4, which are published as supporting information on the PNAS web site. Here, the transitions clearly show hysteresis. This hysteresis indicates that the transitions should occur in two steps. The initial step mainly involves twist changes, while...
differences in the structure and interactions are essential for given new insights into understanding how biological supramolecular models (30–33) and by coarse-grained models (34, 35) have responded to subunit 5 if viewed from subunit in subunit protofilament. If D107 in subunit 0 forms a salt bridge with R52 suggested that R52 is a key residue in this propagation along the to L-type or vice versa along the protofilament is expected. It is used, the various energy terms responsible for folding were implicitly assumed that an elementary event of supercoil transition does not involve any cooperative motion of numerous helical steps. This assumption is endorsed by the agreement between the experimentally and computationally observed order of magnitude of torque necessary to induce this transition. Interestingly, the transient supercoil structures observed in left- to right-handed supercoil transformations here also have been experimentally observed as transient states (9): the experimentally observed structure shown by the triangle in Fig. 1g was observed computationally when insufficient torque was applied, and that indicated by the square was observed after the transition but before energy relaxation.

To induce the conformational transition of each elementary helical step from normal to semicoil, a torque of $3.0 \times 10^2$ pNnm (= 43 kcal/mol radian) carries out a work of 0.8 kcal/mol as the step is twisted by 0.018 radian in this process. When supercoil transition from normal to semicoil occurs, six salt bridges are lost per elementally helical step. These losses should be accompanied by a free energy increase of several kcal/mol. Concomitant transformation of two subunits from L- to R-type should be accompanied by a free energy decrease. Balance between these two competing energy terms results in the above free energy difference of 0.8 kcal/mol. This result is a clear example of a very general principle of the molecular mechanisms for biomacromolecular machinery to function. Functions of biological macromolecules are generally performed by transitions between multiple-energy-minimum substates realized in the native or near-native states of the machinery (25, 26). Such multiple energy minima are brought forth by the existence of competing energy terms or frustration. Frustration exists generally behind energy minima are brought forth by the existence of competing energy terms or frustration. Frustration exists generally behind.

In the dynamic process of supercoil transition at the molecular level, a certain mechanism of propagating the transition from R- to L-type or vice versa along the protofilament is expected. It is suggested that R52 is a key residue in this propagation along the protofilament. If D107 in subunit 0 forms a salt bridge with R52 in subunit +16, the same R52 is also expected to form hydrogen bonds with N132 in subunit +11 because subunit +16 corresponds to subunit 5 if viewed from subunit +11.

Recently, computer simulations carried out by atomic detailed models (30–33) and by coarse-grained models (34, 35) have given new insights into understanding how biological supramolecular structures work. In this study, we utilized the former because fine differences in the structure and interactions are essential for detailed understanding of the supercoiling mechanism. However, coarse-gained models also should be constructed to investigate more macroscopic dynamical behaviors of the polymorphic supercoil transition. We believe that multiscale simulation, a combination of explicit model and coarse-grained models, will become a powerful tool in understanding the dynamic behaviors and functional mechanisms of supramolecular systems.

Materials and Methods

MD Starting from R-Type Structure. MD simulation was performed by using the module SANDER of the molecular simulation package AMBER7 (36) with the parm99 force field. As the initial filament model, the atomic model of the R-type straight filament composed of mutant flagellin A449V from strain SJW1655 of S. typhimurium, consisting of 44 flagellin subunits (316,668 atoms), was placed in a periodically repeated rectangular box of 261 × 261 × 381 Å$^3$, and the gaps were filled with 689,089 water molecules and 528 chloride ions. Chloride ions were added to neutralize the system. Periodic boundary conditions were used, and nonbonded interactions were calculated by Particle-Mesh Ewald method. For the WT (SJW1103) and L-type (SJW1660, i.e., G426A) filaments, simple amino acid substitutions were performed for the pertinent side chains. Total numbers of atoms in the A449V, G426A, and WT filaments were 2,384,463, 2,384,331, and 2,384,199, respectively. Periodic boundary conditions were used, and nonbonded interactions were calculated by the Particle-Mesh Ewald method. During the simulations described in this work, the filament was separated from its images by at least 4–10 layers of water molecules. For each system, a 1.2-ns equilibrium simulation was performed. The first 250-ps simulation was performed with constraints on the initial structure, and the system was equilibrated in an isothermal-isobaric ensemble at 300 K and 1 atm (1 atm = 101.3 kPa).

Construction of Long Filament and Assignment of R- or L-Type of Interface. For each instantaneous structure observed in the simulation, we focused our attention on relative spatial arrangement (translation and orientation) between the two central 11 subunits (elementary helical step). When this arrangement is repeated many times, a helical structure is generated. Twist and curvature of such a helix is defined as the local twist and curvature of the elementary helical step. If these two parameters fluctuate around those of a theoretically expected supercoil structure deduced by the bistable protofilament model (11–14), e.g., 2R/9L (normal) state, the two shortest protofilament interfaces are assigned to R-type and the other nine protofilament interfaces to L-type.

Generating Various Supercoil Structures. To generate metastable supercoil structures, force–bias MDs were performed starting from equilibrated structures of the WT at 900 ps. A torque was applied to the two sets of 11 subunits at both ends of the short filament model in opposite directions to twist the filament from right- to left-handed supercoils around the tangential directions of local axes of these subunits defined for each instantaneous structure. Force–bias MDs were carried out for 25–50 ps and then continued without a torque to equilibrate the system for 50–250 ps. This process was repeated up to 10 times. In the first four iterations, tangential forces were applied to atoms in domains D0 and D1 of these subunits amounting to a torque of $5.0 \times 10^5$ pN-nm. To speed up the equilibration of domains D2 and D3 exposed to solvent, $10.0 \times 10^5$ pN-nm was applied to all of the residues in the rest of the iterations. A total of 37 structures with different supercoil parameters were generated. Equilibrium MD without force bias was initiated from each of these structures. In 18 cases, the filament structures appeared trapped in metastable states relatively quickly. In these cases MDs were extended up to 250 ps. In the other 19 cases...
where relatively large conformational changes continued, MDs were stopped at ~50–200 ps. After 250-ps MD simulations starting from 18 different structures, the filament structures were trapped in metastable states ranging from 1R/10L to 6R/4L: one 1R/10L (small helix amplitude), two 2R/9L (normal), three 3R/8L (coil), three 4R/7L (semicoil), one 5R/6L (curly I), four 6R/5L (curly II), and one 7R/4L. Simulations were further extended for 200–400 ps (in total 2.8 ns) starting from 10 selected structures (three 1R/10L, one 2R/9L, two 3R/8L, three 4R/7L, and one 5R/6L). Finally four 2R/9L (normal), two 4R/7L (semicoil), three 5R/6L (curly I), and one 6R/5L (curly II) structures were identified as metastable states. Total simulation time in these processes was 13 ns.

In the real flagellar filament, elementary helical steps along the filament should, of course, microscopically differ from each other. To generate a realistic flagellar filament structure from the result of simulation, elementary helical steps in each cluster in the twist–curvature space are chosen randomly and stacked repeatedly by superimposing the lower 11 subunits of the first elementary helical step to the upper 11 subunits of the second step.

Observation of Supercoil Transition. Supercoil transitions from right- to left-handed and from left- to right-handed were simulated by using the same force–bias MD described above. For this purpose, a torque ranging from 1.0–10.0 × 10^2 pN nm was applied only to domains D0 and D1. For these simulations, 3.5-ns MD was performed.

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