Specific Arrangement of \(\alpha\)-Helical Coiled Coils in the Core Domain of the Bacterial Flagellar Hook for the Universal Joint Function

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SUMMARY

The bacterial flagellar hook is a short, highly curved tubular structure connecting the rotary motor to the filament acting as a helical propeller. The bending flexibility of the hook allows it to work as a universal joint. A partial atomic model of the hook revealed a sliding intersubunit domain interaction along the protofilament to produce bending flexibility. However, it remained unclear how the tightly packed inner core domains can still permit axial extension and compression. We report advances in cryoEM image analysis for high-resolution, high-throughput structural analysis and a density map of the hook that reveals most of the secondary structures, including the terminal \(\alpha\) helices forming a coiled coil. The orientations and axial packing interactions of these two \(\alpha\) helices are distinctly different from those of the filament, allowing them to have a room for axial compression and extension for bending flexibility without impairing the mechanical stability of the hook.

INTRODUCTION

Bacteria have evolved a sophisticated machine called the flagellum to swim toward more favorable environments from less favorable ones (Macnab, 2003; Berg, 2003; Kojima and Blair, 2004; Sowa and Berry, 2008; Minamino et al., 2008). The flagellum is a macromolecular assembly composed of about 30 different proteins in copy numbers from a few to a few tens of thousands (Namba and Vonderviszt, 1997). It can be divided into three parts: the basal body, spanning the cell envelope to work as a rotary motor as well as a protein export machine; the long filament, extending into the cell exterior to function as a helical propeller; and the hook, working as a universal joint connecting the motor to the helical propeller. The rotary motor is powered by proton motive force across the cell membrane and rotates at around 300 Hz (Kojima and Blair, 2004). The filament is a helical assembly of a single protein, FliC (flagellin), and a few tens of thousands of FliC molecules form a 10–15 \(\mu\)m long, gently curved, relatively rigid, supercoiled tubular structure to be a helical propeller (Namba and Vonderviszt, 1997), which is rotated by the motor to produce thrust for bacterial swimming (Berg and Anderson, 1973; Silverman and Simon, 1974). Poly-morphic transitions of the filament between left- and right-handed helical forms allow bacteria to switch their swimming mode between run and tumble for chemotaxis and thermotaxis (Asakura, 1970; Macnab and Ornston, 1977; Turner et al., 2000). The hook is a short, highly curved tubular structure built by helical assembly of about 120 copies of a single protein, FlgE (DePamphilis and Adler, 1971a; DePamphilis and Adler, 1971b; Kagawa et al., 1979; Wagenknecht et al., 1981). The length of the hook is relatively well regulated to 55 ± 7 nm (Hirano et al., 1994), and its highly curved structure and bending flexibility enable it to work as a universal joint, transmitting motor torque to the filament regardless of the filament orientation off-axis of the motor during run and tumble for taxis.

A previous structural study of a straight hook by electron cryo-microscopy (cryoEM) and helical image reconstruction (Shaikh et al., 2005) revealed that FlgE is composed of three domains (D0, D1, and D2) that are radially arranged from the inner to the outer part of the tubular structure of the hook. A partial atomic model of the hook was built by docking the X-ray crystal structure of a 31 kDa fragment of FlgE (FlgE31) produced by truncation of both terminal chains into the cryoEM density map (Shaikh et al., 2005; Samatey et al., 2004). The model revealed a relatively loose axial packing of subunits in the outer part of the tube made of domains D1 and D2, which appeared to explain the bending flexibility. A molecular dynamics simulation of axial extension and compression of the hook protofilament, which is a longitudinal array of subunits along the 11-start helix, demonstrated that intersubunit bonding interactions between domains D1 and D2 go through multiple steps of changes in bonding partners, realizing a mutual sliding between these two domains to allow extension and compression of the protofilament by about 9 Å to produce high bending flexibility of the hook (Samatey et al., 2004).

In contrast, the D0 domains, which form the inner core of the tubular structure, seemed tightly packed in all directions in the cryoEM density map (Shaikh et al., 2005), which would be reasonable considering that these interactions are the only ones remaining to confer mechanical stability on the hook structure. However, because the protofilament made of the D0 domains in the inner core of a highly curved hook still has to go through a significant level of axial extension and compression by 3–4 Å during the universal joint motion, albeit significantly less than that of the outer part because of the small radial distance from the tube axis, it remained unclear how these tightly packed...
D0 domains could possibly accommodate themselves to such structural perturbations.

Proteins that form the axial tubular structures of the flagellum, such as the rod, the hook, and the filament, all have the heptad repeats in their terminal regions with hydrophobic amino acids at the first and the third or fourth positions within each repeat, which implies the presence of α-helical coiled coil (Homma et al., 1990; Sajo-Hamano et al., 2004). CryoEM studies of the filament structure indicated that the terminal regions of FlgC are located in the inner core (Mimori-Kiyosue et al., 1997), and the atomic model of the filament showed that the inner core tube is indeed formed by axially aligned α-helical coiled coils formed by the N- and C-terminal chains of about 30 residues and that they are tightly packed in all directions by hydrophobic interactions, clearly indicating that these interactions are responsible for the mechanical stability and rigidity of the filament (Yonekura et al., 2003). Although the structural studies of the hook also suggested that its inner core tube is formed by tightly packed domains of an α-helical coiled coil (Shaikh et al., 2005; Samatey et al., 2004), the resolution of the cryoEM image reconstruction (Shaikh et al., 2005) was not high enough to resolve the structures of the terminal α helices. Salmonella FlgE is composed of 402 amino acid residues, and the crystal structure of the FlgE31 fragment contains residues 71–369, which lack the 70 N-terminal and 33 C-terminal residues because of their removal for crystallization (Samatey et al., 2004). That is also why the inner core structure of the hook (domain D0) still remained unknown.

Here we report a cryoEM 3D image reconstruction of the hook at 7.1 Å resolution. Even though we collected images manually, we were able to obtain this relatively high-resolution 3D image within just five days from data collection to the final 3D image reconstruction, by advances in various aspects of cryoEM techniques as we describe below. The structure clearly reveals most of the secondary structures of FlgE, including two terminal α helices forming a coiled coil in the inner core domain. Interestingly, the orientations and axial packing interactions of these two α helices are distinctly different from those of the filament, allowing them to produce a room for axial compression and extension of the protofilament for the bending flexibility of the hook without impairing the mechanical stability of the hook.

RESULTS

High-Contrast, High-Throughput CryoEM Imaging for High-Resolution Structural Analysis

CryoEM methods for studying structures of biological macromolecular assemblies in their native, frozen hydrated forms have rapidly advanced over recent years (Zhou, 2008). The methods of single particle image analyses have become powerful enough to reach a resolution of around 4 Å, resolving main chains and large side chains of proteins in relatively large icosahedral viruses (Yu et al., 2008; Zhang et al., 2008). A similar method has also been applied to tobacco mosaic virus (Sachse et al., 2007), a well-ordered filamentous structure with a well-defined helical symmetry, and the resulting 3D density map also showed well-resolved main chain and large side chains of tobacco mosaic virus coat protein (Sachse et al., 2007). However, it still takes a fair amount of time from image data collection to computational image analysis; it normally takes months or sometimes a year to obtain such high-resolution 3D density maps, because the method requires approximately a million or even larger number of high-quality images of structural subunits to be collected, aligned, and averaged to increase the signal-to-noise ratio of structural information, thereby increasing the resolution of 3D image reconstruction.

We too have put our effort into improving various aspects of the cryoEM technique for high-throughput, high-resolution image analysis of biological macromolecular assemblies. In our previous studies, we used an electron cryomicroscope with a field emission electron gun operated at an accelerating voltage of 300 kV and a liquid helium-cooled top entry specimen holder (JEM-3000SFF, JEOL) to obtain a 3D density map of a straight-type flagellar filaments at 4–5 Å resolution by the conventional Fourier-Bessel method and built a complete atomic model (Yonekura et al., 2003). We believe that extremely low specimen temperatures below 10 K greatly helped high-resolution image data collection by reducing radiation damage significantly more than those recorded at around 100 K by liquid nitrogen cooling to preserve high-resolution structural information (Fujioyoshi et al., 1991). However, it took us over a few years to achieve such a high resolution because the fraction of high-quality images within the data set we collected was often less than a few percentage points, making the data collection extremely slow, and we also had to improve various aspects of image analysis methods to align and average molecular images as accurately as possible.

For the present study, we used a similar type of electron cryomicroscope but additionally equipped it with an Ω-type in-column energy filter (JEM-3200FSC, JEOL) and a 4 x 4 slow-scan CCD camera with a relatively fast readout capability (5 s/frame) (TemCam-F415MP, TVIPS). We operated the microscope at an accelerating voltage of 200 kV, instead of 300 kV, for relatively small specimens with the size less than 300 Å to obtain a higher image contrast. The Ω-type energy filter and the CCD camera gave us a significant improvement in image contrast as well as in data collection efficiency and allowed us to visualize the 3D structure of a self-assembled DNA tetrahedron nanostructure with an edge length of 70 Å and a molecular weight of only 78 kDa at a level of resolution at which the major groove of the DNA double helix is resolved. The 3D density map thus obtained made it possible to discriminate one of the two possible stereoisomer structures over the other (Kato et al., 2009). The effect of the Ω-type energy filter on cryoEM images of the hook is demonstrated in Figure 1 by comparing two images: one with the filter on and the other off.

We were able to optimize the condition for quick freezing of specimens by using Vitrobot (FEI) to make the thickness of vitreous ice film over carbon holes as thin as possible to obtain a better image contrast. We also optimized the specimen temperature over a range between 4.2 K and 80 K to be able to collect images at the best possible contrast with the least radiation damage. We found that the optimization of conditions for sample preparation and image data collection, as well as the additional hardware features of the microscope, gave us a significant gain in the image contrast over the previous ones we used to obtain. The gains in the image contrast are as follows: about 1.8 times by energy filtering; about 1.6 times by controlling ice
recording images at 50 K to 60 K with a high-speed, 4k pixel slow-scan CCD camera gave us an opportunity to explore further technical improvements in image analysis toward high-resolution, high-throughput cryoEM 3D image reconstruction of biological macromolecular assemblies, particularly with helical symmetries, as we describe below for the structural analysis of the flagellar polyhook.

3D Helical Image Reconstruction of the Polyhook

The previous cryoEM structural analysis of the flagellar polyhook was performed by use of a conventional Fourier-Bessel method for helical image analysis (Shaikh et al., 2005). We used the IHRSR method, which is a kind of single particle image analysis method that still utilizes the helical symmetry of the filamentous particle (Egelman, 2000). The conventional Fourier-Bessel approach requires high-quality diffraction patterns showing sharp, well-defined layer line reflections in the Fourier transform of images for image alignment and average for 3D image reconstruction. The filamentous particles have to have well-ordered structures, and the images of the particles have to be straight to give rise to sharp layer lines. One has to select only images without visible bend, which makes the data collection efficiency low, and one still must perform unbending to make layer lines as sharp as possible, which sometimes introduces artifacts. In the IHRSR method, because we segment an image of a long filamentous particle into small images and perform image alignment by treating each segmented image as a single particle image, unbending of the image is done automatically without manual intervention. Once appropriate parameters for image analysis are correctly obtained and set up, including defocus and astigmatism of the images and structural parameters (e.g., the helical symmetry and the axial repeat distance as the initial parameters to be refined in the iterative procedures of image alignment and average), the whole process of image analysis can be done automatically and quickly by parallel processors of a PC cluster computer. We used the helical symmetry and the axial repeat distance obtained by the previous study of the polyhook as the initial parameters to be refined, but these structural parameters can be easily obtained from the Fourier transforms of the images as well. We were able to obtain a 3D image reconstruction at a resolution of 7.1 Å (based on the Fourier shell correlation [FSC] = 0.5 criterion, 5.4 Å with the FSC = 0.143; see Figure S2), and the whole process of structural analysis from image data collection to final 3D reconstruction was done within five days. The density map now clearly resolves the four radially arranged domains of FlgE, D0, Dc, D1, and D2, and secondary structures, such as α helices, β sheets, β-hairpins, and even loops in these domains (Figures 2 and 3), giving us deep insights into how the molecular structure and intersubunit packing interactions are designed to confer the structural characteristics, such as bending flexibility, twisting rigidity, and mechanical stability, on the hook for the universal joint function.

Terminal α Helices in the Inner Core Domain D0

The density map now clearly shows two rod-shaped densities in the inner core domain (D0) of the tubular structure of the hook (Figures 2 and 3). These densities occupy about a half of the portion for which detailed structural information was not available in the previous study (Shaikh et al., 2005; Samatey et al., 2004). These rod-shaped densities most likely correspond to the terminal α helices forming a coiled coil, as predicted. One of the two rod-shaped densities is shorter than the other, facing the central channel with a larger tilt. The shorter one is 36 Å long and the longer one is 54 Å long. If these densities are both entirely α helix, the 1.5 Å axial repeat per residue of α helix converts these lengths to 24 and 36 residues for the shorter and longer ones, respectively. The secondary structure prediction from the amino acid sequence of FlgE shows that the N- and C-terminal α helices are 24 and 36 residues long, respectively (Figure S3), giving good agreement with the lengths of the rod-shaped densities, indicating that the shorter one is the N-terminal α helix and the longer one facing the central channel is the C-terminal one (Figures 2B and 3).

The α-helical coiled coil structure is predicted because the N- and C-terminal regions of FlgE, like all the other flagellar axial proteins, have the heptad repeat with hydrophobic residues at the first and the third or fourth positions within the seven-residue repeat (Homma et al., 1990; Saijo-Hamano et al., 2004). Such hydrophobic residues are likely to interact with each other at the interface of α helices in the coiled coil structure. We therefore placed the two terminal α helices of FlgE in the density map under such restriction to build an atomic model (α helices in blue and red in Figures 2A, 2B, and 3).

Structure of the Two Outer Domains

The density map also shows almost all the secondary-structural elements, such as β sheets and β-hairpins, clearly in the outer...
two domains D1 and D2 as well (Figure 3). Even the triangular loop (indicated by arrowhead in Figure 3), which was identified to play an important role in the intersubunit sliding interactions between domains D1 and D2 for the universal joint function of the hook, can be discerned. These fine structural features allowed us to determine the positions and orientations of the atomic models of these two outer domains far more accurately than in the previous study (Shaikh et al., 2005; Samatey et al., 2004). We docked the atomic models of the two domains separately into the density map, connected them by two chains, and refined the whole model under stereochemical restraints by using the MDFF program (Trabuco et al., 2008).

We compared this new model with the previous one based on a lower resolution map (Shaikh et al., 2005; Samatey et al., 2004). Although the positions and orientations of domain D2 are almost the same, the orientations of domain D1 are significantly different (Figure S4). In the new model, the major axis of domain D1 is less tilted by ∼14° within the radial plain, and the angle between the major axes of domains D1 and D2 is narrower by ∼17°. Because the intersubunit interactions between domains D1 and D2 are not so different from the one identified in the previous model (Samatey et al., 2004), the mutual sliding interactions at this intersubunit interface we observed by molecular dynamics simulation (Samatey et al., 2004) would still be valid. However, the results of this comparison indicate the risk of overinterpretation of atomic models built by docking crystal structures of the subunits into low-resolution density maps of macromolecular complexes obtained by cryoEM image analysis. Although the atomic model was carefully refined in the previous study (Shaikh et al., 2005; Samatey et al., 2004) by a real-space model refinement program (Chen et al., 2003), the accuracy of the domain positions and their orientations was inevitably limited because of the relatively low resolution of the previous map, which was probably around 15 Å.

**Polypeptide Chains Folded Within Domain Dc**

Of the N-terminal 70 residues and the C-terminal 39 residues that are missing in the FlgE31 crystal structure, we already modeled the N-terminal 24 and the C-terminal 36 residues as α helix in domain D0. Therefore, in the remaining two regions, 46 residues of Asn-25–Arg-70 and three residues of Asn-364–Asp-366, 49 residues are folded within the portion between domains D0 and D1 (Figures 2B and 3), which we named domain Dc to describe the structure, although domains D0 and Dc may actually form one domain. It is, however, still difficult to build the atomic model for domain Dc because the resolution of the present density map is still limited (Figures 2A, 2B, 3, and 4C) and also because only one short α helix is predicted for eight residues of Thr-35–Phe-42 in the N-terminal region (Figure S3). Because domain Dc contains rod-shaped densities (Figure 4C), the short α-helix may be assigned to one of them, but unambiguous assignment was not possible.

Interestingly, the three residues from Asn-364 to Asp-366 are too short to connect the C-terminal gap of about 40 Å between Ser-363 and Leu-367 in our present model—namely, the C terminus of the crystal structure and the N terminus of the 36-residue C-terminal α-helix. Even a relatively extended chain requires at least a dozen residues to connect the gap of 40 Å. We therefore carefully looked at the atomic model of domain

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**Figure 2. Three-Dimensional Density Maps of the Polyhook with a Fitted Atomic Model**

(A) End-on view from the distal end. The diameter of the hook is 180 Å and that of the channel is 18 Å. Scale bar, 50 Å.

(B) Side view of a longitudinal section through the axis. Radial ranges of the four domains are indicated at the bottom.

(C) Side view showing the surface.
D1 docked into the density map and found that its C-terminal, seven residues Asn-357–Ser-363, which forms the last β strand of FlgE22 Å in length at the edge of the β sheet (a black ribbon in Figure 3), is clearly out of density and that these seven residues are not involved in the hydrophobic core of domain D1. Thus, this β strand may be an artifact produced by terminal truncation of FlgE to produce FlgE31 and/or crystallization of FlgE31. It is therefore quite likely that these seven residues extend in the almost opposite direction toward domain D0, specifically toward Leu-367 at the N terminus of the C-terminal α-helix, and, together with the remaining three residues (Asn-364–Asp-366), the 10-residue chain (Asn-357–Asp-366) goes through the upper part of domain Dc and connects to the C-terminal α-helix over a gap of 27 Å between Asn-357 and Leu-367 (Figure 3).

### Intersubunit Domain Interactions for Bending Flexibility, Twisting Rigidity, and Structural Stability

Cylindrical surface arrays of the four domains at their different radial positions show their intermolecular domain packing interactions as presented in Figure 4, for which subfigures were made by removing each domain at a time from the outside. The full hook structure in Figure 4A shows the strongly connected, continuous helical arrays of D2 domains along the 6-start helix on the outer surface of the hook with a wide gap between them, indicating that a highly bent structure of the hook can be easily generated. The removal of D2 domains reveals the array of D1 domains (Figure 4B), which shows that the packing of D1 domains is loose in all directions, also permitting highly flexible bending of the hook.

Further removal of D1 domains shows the shape of domain Dc, which looks like a capital letter “H” tilted to the left, and its cylindrical surface packing (Figure 4C). The Dc domains are connected along the left-handed 5-start helical arrays with a large enough gap between them to allow flexible bending of the hook. These left-handed helical arrays of Dc domains (Figure 4C) and the right-handed 6-start helical arrays of D2 domains (Figure 4A) at the outer radius together form a meshlike
structure, which must be responsible for the twisting rigidity as well as the structural stability of the hook to transmit the motor torque to the helical propeller for its high-speed rotation and frequent reversal. Finally, the removal of all the outer domains leaves the two terminal \( \alpha \) helices forming a coiled coil in the inner core domain D0, and the extensive intermolecular packing interactions between D0 domains indicate that they are responsible for structural and mechanical stability of the hook (Figure 4D). Thus, the intersubunit domain connections along different helical lines at different radial positions hold the entire hook structure together while the axial gaps between these helically connected domains allow the hook to have a relatively large bending flexibility.

**Extensibility and Compressibility of the Inner Core Tube**

According to a simple geometric calculation, the axial array of D0 domains in the inner core still has to go through a significant level of axial extension and compression by 3–4 Å during the universal joint motion of a highly curved hook, although this is significantly less than those of the outer domains as a result of the small radial distance from the tube axis. Therefore, it was puzzling how these tightly packed D0 domains could possibly accommodate themselves to such structural perturbations. Now the structural design of the inner core reveals the mechanism (Figure 5A). Because the \( \alpha \)-helical coiled coil motif of the terminal regions are shared by all the flagellar axial proteins including FliC and FlgE (Homma et al., 1990; Saijo-Hamano et al., 2004), the structure of the inner core of the hook was thought to be similar to that of the filament structure, which shows a tight axial packing of the axially extended terminal \( \alpha \) helices of FliC for structural and mechanical stability of the filament (Figure 5B) (Yonekura et al., 2003). However, the terminal \( \alpha \) helices of FlgE are significantly more tilted than those of FliC, with the longer, C-terminal one more tilted than the N-terminal one, giving rise to an axial gap of about 5 Å between subunits with an overlap of about 10 Å between the two ends of the C-terminal \( \alpha \)-helix (Figure 5A), giving enough space for compression and stable intermolecular interactions at the same time. Thus, the structure and packing arrangement explains how D0 domains of the hook allow the extension and compression of their axial array over 3–4 Å while maintaining the mechanical stability of the entire hook structure.

**DISCUSSION**

**Similarity and Difference between the Hook and the Filament**

Now we have a fairly reliable atomic model of the hook structure for 348 residues out of 402 residues of FlgE, which is about 87% of the molecule, on the basis of the cryoEM density map at around 7 Å resolution and the crystal structure for residues 71–357. The structure reveals four radially arranged domains—D0, Dc, D1, and D2—and their intermolecular interactions, all of which show quite distinct characteristics from those of the filament structure, despite the similarity in their quaternary structures forming supercoiled tubular structures composed of 11 protofilaments with similar helical symmetries for subunit arrangements. The filament can be in multiple forms of supercoils with relatively small but well-defined curvatures and twists by its unique polymorphic supercoiling mechanism (Asakura, 1970; Calladine, 1975, 1978; Kamiya et al., 1979) and shows a limited flexibility in bending by mechanical perturbations during the propeller motion (Hoshikawa and Kamiya, 1985). This is the basis of polymorphic supercoiling of the filament. The hook also shows polymorphic transformations of its supercoiled forms (Kato et al., 1984). However, the hook is highly curved with an order of magnitude smaller radius of curvature than those of the supercoiled filaments and has a large bending flexibility with an apparently continuous variation in the curvature from the stably supercoiled form in the absence of any mechanical perturbations to an almost straight form as typically seen in electron micrographs of negatively stained specimens of the flagellar hook-basal body with the filament attached. Because the filament is relatively rigid to be a helical propeller and the hook must be highly flexible in bending for its universal joint function, their structural designs should be quite different from each other to confer these distinct mechanical characteristics on each of them. However, the structural mechanisms that give rise to these distinct mechanical properties remained unclear because of the limitation in high-resolution structural information available from the previous studies (Shaikh et al., 2005; Samatey et al., 2004).
The recent advances in electron cryomicroscopy and image analysis allowed us to see the fine details of the structure to solve most of the puzzles within a relatively short time as described in Results. It is interesting to see that an apparently similar structural motif of $\alpha$-helical coiled coil formed by the terminal chains of FlIC and FlGE can be oriented and packed differently to produce these two distinct structural designs for distinct mechanical functions.

Although the curvatures of the supercoiled filaments are formed along the 11-start protofilament (Asakura, 1970; Calladine, 1975), those of the supercoiled hook are not along the 11-start (Kato et al., 1984). It has been proposed on the basis of the atomic model of a supercoiled form of the hook that the supercoiled forms are produced by close packing interactions between the right-handed 6-start connections of D2 domains on the inner side of the curved hook. A few sets of distinct D2-D2 interactions can define a limited number of virtual protofilament directions to produce distinct supercoils that have been observed. With the more accurate atomic model of the straight hook obtained by the present study, we should be able to identify amino acid residues that are involved in such D2-D2 interactions.

**Future Advances in CryoEM Image Analysis**

Structures of biological macromolecules are fine-tuned for their specific functions over evolution, and that is more so for large molecular assemblies. The structural difference between the hook and filament is a clear example demonstrating how a subtle difference in the structural designs can be used to produce such distinct mechanical functions. This also means that direct visualization of the structures of macromolecular assemblies at sufficiently high resolution is essential for the understanding of the mechanisms. Because so many interesting biological functions driving various cellular activities are all based on either temporary or stable interactions within and between macromolecular assemblies, direct visualization of their structures at work is no doubt essential for the mechanistic understanding. CryoEM image analysis would be the only tool available for such an approach.

Various technologies for cryoEM image analysis have shown remarkable advances in reaching high resolution, and in particular, single particle image analyses of relatively large virus particles have shown the power to resolve main chains and large side chains of proteins at resolutions slightly better than 4 Å (Yu et al., 2008; Zhang et al., 2008; Sachse et al., 2007). However, the whole process of structural analysis from image data collection to final 3D image reconstruction still tends to take a fairly long period of time and requires substantial efforts. Efforts have been made to improve the throughput of high-resolution cryoEM image analysis by automated data collection and image analysis, and a recent progress is remarkable (Stagg et al., 2008). We also demonstrated here that, with various improvements in electron microscope hardware, methods of frozen-hydrated sample preparation, and imaging conditions, we can gain almost five times higher image contrast than those conventionally obtained, allowing us to reach resolution high enough to resolve most of the protein secondary structures within a few days. An encouraging aspect of cryoEM image analysis is that there is still a large room for further improvements to make the method much more powerful in achieving even higher resolution within a shorter time. We therefore foresee a bright future in the structural and molecular biology.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation and Electron Microscopy**

Bacterial flagellar polyhooks were isolated as described elsewhere (Aizawa et al., 1988). We used a polyhook strain of Salmonella enterica serovar Typhi-
merium, SJW880. The polyhook solution was incubated at 4°C overnight before freezing to turn the supercoiled form into straight. A 3.3 μl sample solution was applied onto a Quantifoil holly carbon molybdenum grid (R0.6/1.0, Quantifoil Micro Tools GmbH, Jena, Germany) and was plunge-frozen into liquid ethane using a fully automated vitrification device (Virotect, FEI). The specimen was observed at temperatures of 50–60 K using a JEOL JEM3200FC electron microscope, which is equipped with a liquid helium-cooled specimen stage, an Ω-type energy filter, and a field-emission electron gun operated at 200 kV. Zero energy-loss images, with a slit setting to remove electrons of an energy-loss larger than 10 eV, were recorded on a 4 k x 4 k 15 μm/pixel slow-scan CCD camera. TemCam-F415MP (TVIPS) at a magnification of 89,000, a defocus range of 0.5–2.0 μm, and an electron dose of ~20 electrons/Å². The magnification was calibrated by measuring the layer line spacing of 23.0 Å in the Fourier transform of images of tobacco mosaic virus mixed in the sample solution. The image pixel size at this magnification was 1.68 Å/pixel. In total, 310 CCD images were collected manually in one day and used for image analysis.

**Image Analysis**

Defocus and astigmatism in the image was determined using CTFFIND3 (Mandell and Grigorieff, 2003). Images of the polyhook from 310 CCD frames were boxed into 86,444 segments of 512 x 512 pixels with a step shift of 10 pixels along the helical axis using EMAN’s boxer program (Ludtke et al., 1999). The in-plane orientation of each polyhook was retained in the segment and recorded in a list to avoid interpolation when rotating the image. Images were then phase-corrected by multiplying a phase and amplitude contrast transfer function (CTF) with the astigmatism obtained by CTFFIND3 (Mandell and Grigorieff, 2003). We used a ratio of 7% for the amplitude CTF to the phase transfer function (CTF) with the astigmatism determined by CTFFIND3 (Mandell and Grigorieff, 2003). This procedure for the CTF correction results in the multiplication of the square of the CTF (CTF²) to the original structure factor and suppresses the noise around the nodes of the CTF, allowing more accurate image alignment. The amplitude modification by CTF² will be corrected in the last stage of image analysis as described later. The images were then high-pass filtered (285 Å) to remove a low spatial frequency density undulation, normalized, and cropped to 320 x 320 pixels. Further image processing was performed with the SPIDER package (Frank et al., 1996) on a PC cluster computer with 40 CPUs (RC server Calm2000, Real Computing, Tokyo, Japan). A series of reference projection images was generated for each reference volume by rotating the volume azimuthally about the filament axis between 0° and 360° and projecting the volume every 2° to produce all views. The raw images of the boxed polyhook segments were translationally and rotationally aligned and cross-correlated with the set of reference projections to produce the following information: an in-plane rotation angle, an x-shift, a y-shift, an azimuthal angle, and a cross-correlation coefficient for each segment. Particles with a small cross-correlation coefficient were discarded. The polarity of the particles was tracked with respect to their respective filament. Even with our high-contrast imaging technique, the orientation of each individual particle was somewhat ambiguous as a result of the relatively low contrast and high noise level of the segment image. Therefore, the orientation was defined as that of the majority of the particles for each filament during each alignment cycle, and all the segments identified to have the opposite orientation were discarded. A 3D reconstruction was then generated by back-projection. The symmetry of this new volume was determined by a least-squares fitting algorithm, and this symmetry was imposed on the reconstruction (Egelman, 2000). The new symmetry-enforced volume was used as a reference for the next round of alignment. This process was repeated iteratively until the symmetry values converged to a stable solution. In the refinement phase, to reduce the noise in the reference and increase the
accuracy of aliment, we used automask2 from EMAN package to impose the edge smooth mask to the reconstruction.

In the analysis, the polarities of the segment images were determined reliably. On average, the in-plane angles for 95% of the segments from a polyhook filament showed the same polarity. We used the structural parameters that were obtained in the previous work (Shaik et al., 2005) as the initial parameters to be refined. The initial parameters were an axial rise of 4.23 Å and an azimuthal rotation angle of 64.78° along the 1-start helix, and they were converged to 4.12 Å and 64.78°, respectively.

The resulting reconstruction was then modified by multiplying the transform of the reconstruction by $1/(1+C\bar{F}\bar{F}^*+1/SNR)$ to compensate for the amplitude distortion by the contrast transfer function. A smooth band-pass filter was applied to the transform of the 3D density distribution to reduce the amplitude by 90% for the resolution range lower than 14 Å and to make it reach zero beyond 6 Å.

SUPPLEMENTAL DATA

Supplemental data include four figures and three movies and can be found with this article online at http://www.cell.com/structure/supplemental/S0969-2126(09)00374-8.

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REFERENCES


