Structural Dynamics of the MecA-ClpC Complex

A TYPE II AAA+ PROTEIN UNFOLDING MACHINE

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Background: The structure of the type II AAA+ hexameric molecular machine is highly dynamic.

Results: Nucleotide binding and hydrolysis induce twisted movements of pore loops and relative rotation between two AAA+ rings.

Conclusion: An allosteric communication between two AAA+ rings is present in type II AAA+ hexamer.

Significance: This work provides a comprehensive understanding of the structural dynamics of type II AAA+ hexamer.

The MecA-ClpC complex is a bacterial type II AAA+ molecular machine responsible for regulated unfolding of substrates, such as transcription factors ComK and ComS, and targeting them to ClpP for degradation. The six subunits of the MecA-ClpC complex form a closed barrel-like structure, featured with three stacked rings and a hollow passage, where substrates are threaded and translocated through successive pores. Although the general concepts of how polypeptides are unfolded and translocated by internal pore loops of AAA+ proteins have long been conceived, the detailed mechanistic model remains elusive. With cryoelectron microscopy, we captured four different structures of the MecA-ClpC complexes. These complexes differ in the nucleotide binding states of the two AAA+ rings and therefore might presumably reflect distinctive, representative snapshots from a dynamic unfolding cycle of this hexameric complex. Structural analysis reveals that nucleotide binding and hydrolysis modulate the hexameric complex in a number of ways, including the opening of the N-terminal ring, the axial and radial positions of pore loops, the compactness of the C-terminal ring, as well as the relative rotation between the two nucleotide-binding domain rings. More importantly, our structural and biochemical data indicate there is an active allosteric communication between the two AAA+ rings and suggest that concerted actions of the two AAA+ rings are required for the efficiency of the substrate unfolding and translocation. These findings provide important mechanistic insights into the dynamic cycle of the MecA-ClpC unfoldase and especially lay a foundation toward the complete understanding of the structural dynamics of the general type II AAA+ hexamers.

Spatially and temporally controlled protein degradation plays an essential role in various cellular regulatory events (reviewed in Refs. 1–3). In bacteria, a large part of this vital process is performed by ClpP protease, the counterpart of the eukaryotic 20 S proteasome core complex (4). Similar to the eukaryotic proteasome, the multimeric ClpP protease also requires an associated regulatory component, such as ClpA, ClpX, or ClpC, in a homo-hexameric form, to recognize, unfold, and translocate specific substrates (reviewed in Refs. 5–8). ClpA, ClpX, and ClpC can also function alone to disaggregate and refold protein aggregates or assemblies (9–12). Together with ClpB, which is a stand-alone ATP-dependent chaperone (13), they belong to the HSP100/Clp family of the AAA+ superfamily proteins and can be categorized into two types, consisting of one (ClpX) or two (ClpA, ClpB, and ClpC) nucleotide-binding domains (NBD),5 namely type I and type II AAA+ enzymes, respectively.

HSP100/Clp proteins form ring-like hexamers with a central chamber. Inside the hexameric complex, the diameter of the chamber is restricted by multiple layers of axial loops. These loops, inserted at different positions of the NBDs, are indispensable for substrate binding, unfolding, and translocation through successive central pores and eventually the delivery to ClpP in case of degradation (14–17). Over the last 2 decades, numerous efforts have been devoted to the structural determination of the functional hexameric structures of the HSP100/Clp proteins, aiming at the mechanistic elucidation of substrate

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5 The abbreviations used are: NBD, nucleotide-binding domain; CTD, C-terminal domain; NTD, N-terminal domain; ITC, isothermal titration calorimetry; PDB, Protein Data Bank; ATP γS, adenosine 5’-O-(thiotriphosphate).
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unfolding and translocation within the axial loop-lining chamber. The atomic structure of the hexameric ClpX complex reveals an asymmetric arrangement of the six subunits, with pore loops at axially staggered positions (18), providing the first structural evidence that the axial position of pore loops is likely modulated by nucleotide binding. Together with earlier biochemical data (16, 17, 19–21), the pore loop residues are thought to exert mechanical force on the peptide track of the substrate, in response to ATP hydrolysis, to drive substrate unfolding and translocation (16).

However, our understanding of the mechanistic cycle of the hexameric HSP100/Clp proteins is far from complete, especially for the type II HSP100/Clp family proteins with two NBD rings (named D1 and D2), which add another layer of complexity to biochemical and structural studies. Full-length type II AAA^+ proteins either fail to form hexamers in crystals, such as ClpA (22) and ClpB (23), or only form less ordered hexamers in crystals that exhibit very limited x-ray diffraction, such as ClpC (24). In contrast, truncated type II AAA^+ proteins, e.g. ClpC-ΔD2 (24), p97-ΔD2 (25), and D2-NSF (N-ethylmaleimide-sensitive fusion protein) (26, 27), are capable of forming symmetric hexamers in well diffracting crystals. These results suggest a highly dynamic nature of the full-length proteins in hexameric form. Consequently, despite years of efforts, only one hexameric structure of a type II AAA^+ protein, p97/VCP, has been solved at atomic resolution (28, 29). In the meantime, complementary data from cryoelectron microscopy (cryo-EM) studies of the full-length type II AAA^+ proteins have confirmed the overall hexameric architecture and revealed some new structural features to a few specific examples studied (23, 30–38). Nevertheless, many important aspects regarding the general mechanistic model of the two NBD rings remain to be investigated, including possible concerted actions of NBDs within the same ring, coordination of two NBD rings, and ATP hydrolysis-mediated local conformational changes on NBD domains, as well as structural rearrangements at both the intra- and inter-ring levels.

MecA, as a ClpC-specific adaptor protein, is involved in competence development in Bacillus subtilis through targeting the transcription factor ComK to the ClpCP protease complex for degradation (39). MecA is also required for the assembly (40, 41) and functional activation (10) of the ClpC hexamer. MecA recognizes a specific hydrophobic sequence within its clients, proteins of the full-length MecA and ClpC variants were coexpressed in Escherichia coli BL21 (DE3) cells using vectors pGEX-6P-1 (GE Healthcare) and pBB75 (Novagen), respectively. The MecA-ClpC complexes were purified through a glutathione-Sepharose column (GS4B) (GE Healthcare), and the GST was cleaved by the PreScission protease (GE Healthcare). ComK-GFP was overexpressed in E. coli BL21 (DE3) cells using a PLM303 vector and purified through an amylose resin column (New England Biolabs). The maltose-binding protein tag of ComK-GFP was cleaved by the PreScission protease (GE Healthcare). The MecA-ClpC complexes and ComK-GFP were further purified by anion-exchange (Source 15Q, GE Healthcare) and size-exclusion (Superdex 200, GE Healthcare) chromatography. Purifications were performed at 4 °C throughout. The details of protein preparation were described previously (41, 44). Mass spectrometry analysis of the MecA-ClpC complexes indicates that the residual ADP or ATP level is barely detectable.

Isothermal Titration Calorimetry (ITC)—Calorimetric measurements were performed with an ITC200 microcalorimeter (MicroCal LLC). MecA-ClpC complexes purified as mentioned above were concentrated to ~0.05 mM in a buffer containing 25 mM Tris-HCl, 50 mM KCl, and 5 mM MgCl2 (pH 7.5). The protein variants were titrated at 25°C with 5 mM ATP, which was freshly prepared with the same buffer. The data were analyzed with a one-site binding model using Origin 8.0 software (OriginLab Corp.).

ATPase and Unfolding Assays—The ATPase activities of ClpC variants were determined in the presence of MecA using a coupled spectrophotometric assay (45), following an established protocol (24). The unfolding assay measures fluorescence change of ComK-GFP in the presence of full-length of nucleotides to the NBD, but abolishes its hydrolysis activity. After incubation of the protein variants with ATP or ADP, we determined the cryo-EM structures of four related complexes, namely MM-ATP, MM-ADP, WM-ATP, and MW-ATP. Our cryo-EM maps show that MecA serves as a specificity filter in the substrate entrance to control the access of substrates to the D1 pore loops. We also found that ATP hydrolysis could affect the compactness of the D2 ring and, subsequently, the constellation of the six ClpP-interacting loops. More importantly, comparison of these structures indicates that their D1 pore loops are in different axial and radial positions in these complexes, and the D2 rings undergo a rotational movement to a varying extent. Such a rotation, coupled with observed axial movement of the pore loops, suggests that the pore loops likely drag the substrate in a spiral trajectory to facilitate the substrate unfolding and translocation during an ATP hydrolysis cycle. Additionally, our structural and biochemical data indicate an inter-ring allosteric communication between the two NBD rings and suggest that two rings actively talk to each other and work in a concerted way to fulfill their function.

EXPERIMENTAL PROCEDURES

Purification and the Hexameric Complex Assembly of MecA and ClpC Variants—Proteins of the full-length MecA and ClpC variants (E280A, E618A, K214Q/E618A, E280A/K551Q, E280A/E618A, 489–5G-5A-490, 489–5G-490, 448–499, 448–499, 487–491, 486–492, D345AE346A, R380A, E495A, R528A, R638A, and R650A) were coexpressed in E. coli BL21 (DE3) cells using vectors pGEX-6P-1 (GE Healthcare) and pBB75 (Novagen), respectively. The MecA-ClpC complexes were purified through a glutathione-Sepharose column (GS4B) (GE Healthcare), and the GST was cleaved by the PreScission protease (GE Healthcare). ComK-GFP was overexpressed in E. coli BL21 (DE3) cells using a PLM303 vector and purified through an amylose resin column (New England Biolabs). The maltose-binding protein tag of ComK-GFP was cleaved by the PreScission protease (GE Healthcare). The MecA-ClpC complexes and ComK-GFP were further purified by anion-exchange (Source 15Q, GE Healthcare) and size-exclusion (Superdex 200, GE Healthcare) chromatography. Purifications were performed at 4 °C throughout. The details of protein preparation were described previously (41, 44). Mass spectrometry analysis of the MecA-ClpC complexes indicates that the residual ADP or ATP level is barely detectable.
MecA, ClpC variants, and ClpP. The detailed experimental procedures were described previously (24).

**Cryoelectron Microscopy**—For the MM-ATP, WM-ATP, and MW-ATP complexes, purified protein complexes were diluted with a buffer containing 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂, 2 mM ATP (pH 7.5) and incubated in a water bath at 37 °C for 40 min. For the MM-ADP complex, the sample was diluted with a similar buffer except that ADP was used. In all cases, 4-μl aliquots of samples (~100 nM) were applied to glow-discharged Quantifoil 2/4 grids (Quantifoil Micro Tools GmbH), blotted at 20 °C and 100% humidity, and plunged into liquid ethane using an FEI Vitrobot Mark IV. Cryo-grids were examined using an FEI Titan Krios transmission electron microscope operated at 300 kV. Images were recorded at a nominal magnification of ×59,000 on an FEI Eagle 4 k × 4 k CCD camera under low dose conditions (~20 e⁻/Å²), using the automated data collection system AutoEMation (46).

**Image Processing**—Particle (150 × 150 pixels) picking was done using a method based on locally normalized cross-correlation functions (47), against a three-dimensional template generated from the crystal structure of the MecA-ClpC complex (24). The particles were first subjected to manual verification and then classified using a reference-free classification method with the software Relion (48). Classes of particles showing apparent structural disintegration were excluded from further analysis. The statistics of the four samples are summarized in Table 1. The three-dimensional reconstruction and refinement were performed using SPIDER (49) following standard projection matching protocols (50) with modifications. Specifically, parameters of the contrast transfer function were estimated using SPIDER, and the particles were corrected using a phase-flipping method (50). The initial model for multiple reference matching was derived from the crystal structure of the MecA-ClpC complex (24). The reconstruction was performed using the SPIDER command BP 32F with a C6 symmetry imposed. To prevent possible reference bias, different initial models were tested. During the refinement, the angular step size was changed gradually from 20° to 1° (20, 15, 12, 10, 8, 6, 4, 2, 1.5, and 1°), and each step was iterated 3–5 times until convergence was achieved. The final resolution was estimated using 0.5 cutoff criterion of the Fourier Shell Correlation (Table 1). For further evaluation of the density maps, the local resolution maps (supplemental movie 1) of the four maps were calculated using Bsoft (51), with a box size of 20 pixels. Additional validations were performed by reference-swapping tests, e.g. the low pass filtered WM-ATP map was used as the initial model for the refinement of the MW-ATP particles. After refinement, the new MW-ATP map still converged to the original MW-ATP one. Similar tests were done with other groups of particles.

**Difference Map Analysis**—The cryo-EM maps of the MM-ADP, WM-ATP, and MW-ATP complexes were aligned to the map of the MM-ATP complex using the SPIDER command “OR 3Q.” Four maps were then normalized to a mean of zero and a variance of 1. The difference maps were constructed by subtracting one map from the other using the SPIDER command “SU.”

**Flexible Fitting**—The initial atomic model was based on the crystal structure of the MecA108-ClpC complex (Protein Data Bank (PDB) code 3PX1), which lacks coordinates for three pore loops (residues 243–257, 281–300, 587–600), the D2 VGF loop (residues 665–685), the D1-D2 linker (485–491), and a few additional residues 640–643. To generate a complete atomic model, the coordinates of the pore loops were modeled with MODELLER (52), using the crystal structures of ClpB (PDB code 1BJK) (53) and ClpA (PDB code 1R6B) (54) as templates. The atomic model was first docked into the cryo-EM maps manually, followed by a flexible fitting based on molecular dynamics simulation (55) using the NAMD package (56). To avoid overfitting, both the C6 symmetry restraints (57) and domain restraints were included during the flexible fitting. Specifically, a protomer of the complete hexameric model was divided into seven rigid bodies, including the C-terminal domain (CTD) of MecA, N-terminal domain (NTD), M domain, D1 large subdomain, D1 small subdomain, D2 large subdomain, and D2 small subdomain, of ClpC. Chimera (58) and PyMOL (59) were used for graphic visualization.

**RESULTS**

**Characterization of the Nucleotide Binding Properties of the Two NBD Rings**—There are 12 nucleotide-binding sites in a single MecA-ClpC hexamer. The hexameric HSP100/Clp proteins are thought to display an asymmetric arrangement of subunits for intersubunit communication (18, 20, 31), because of partial nucleotide occupancy (60). It is difficult to quantify the nucleotide occupancies of these sites individually in a wild type hexamer. One can introduce different mutations to the D1 and D2 NBDs, such that the nucleotide affinities to different NBD rings could be estimated separately. For this purpose, we introduced Walker A mutations (K214Q and K551Q) to the WM ClpC constructs, respectively. The Walker A mutation would abolish the nucleotide binding (null mutation, abbreviated as N). The affinities of ATP to three relevant MecA-ClpC complexes, NM, MN, and MM, were assessed by ITC. As shown in Table 2, under our experimental condition with 15-fold excess of ATP, both the NM and MN complexes could take up to six ATP molecules. Furthermore, the affinity of

**TABLE 1**

| Sample name | MM-ATP | MM-ADP | WM-ATP | MW-ATP |
|-------------|--------|--------|--------|--------|
| Initial micrograph number | 1,451 | 1,487 | 1,384 | 2,114 |
| Selected micrographs for particle picking | 530 | 573 | 750 | 994 |
| Particle number used for two-dimensional classification | 71,451 | 48,239 | 52,185 | 129,969 |
| Particle number used for refinement | 36,688 | 26,037 | 41,902 | 45,514 |
| Defocus range (μm) | 3.7–4.0 μm | 1.5–4.0 μm | 1.5–3.0 μm | 1.5–3.0 μm |
| Resolution | 10.0 Å | 9.4 Å | 11.0 Å | 9.0 Å |
| Cross-correlation coefficient of MDFF | 0.83 | 0.84 | 0.84 | 0.82 |

MDFF is flexible fitting based on molecular dynamics simulation.
ATP to the D2 NBDs appears to be 10 times higher than to the D1 NBDs. However, in the case of the MM complex, the calorimetric changes due to the binding of ATP to the D1 NBDs appear to be overshadowed by the strong binding to the D2 NBDs, and only six binding sites could be detected. The detection of the ATP binding to the D1 NBDs in the MM complex might need a much higher concentration of ATP. Highly consistently, ITC experiments on ClpB (61) (a highly conserved homolog of ClpC) have derived exactly the similar conclusions. Therefore, our data and their data suggest that the six nucleotide-binding sites of the D2 ring could be fully occupied easily, and with a large excess of nucleotides all 12 binding sites of the MM complex might be saturated as well.

Accordingly, we incubated the MM, WM, and MW complexes with a large excess of ATP or ADP (2 mM), aiming at locking the two NBD rings in different nucleotide binding states, and applied single particle cryo-EM analysis to four related complexes, namely the MM-ATP, MM-ADP, WM-ATP, and MW-ATP complexes.

**Overviews of the Four Cryo-EM Maps**—Cryo-EM micrographs of the four complexes show a fair distribution of particles, with recognizable end-on views and side views (Fig. 1A). The application of a reference-free classification method (48) to the raw particles revealed nicely resolved two-dimensional class averages, with a wide range of orientations (Fig. 1, B–E), clearly demonstrating the quality of the data. Four cryo-EM maps were reconstructed and refined with the standard reference projection-matching technique (9–11 Å) (Table 1). We chose to keep the C6 symmetry imposed during image reconstruction, which would presumably allow the discovery of relatively large scale conformational changes and identification of the global trends of structural remodeling caused by nucleotide binding and hydrolysis.

As a result, the four EM maps show a similar overall architecture, but display apparent differences in a number of locations, especially at regions where pore loops are situated (Fig. 2). In the cryo-EM maps, the CTDs of MecA are packed tightly with the NTDs of ClpC to form a distal N-ring, such that they appear as a single density lobe even when the maps are displayed at a high threshold (data not shown), confirming the essential contribution of MecA to the oligomerization of ClpC (24, 40). Densities of the ClpC M domains are also well resolved and clearly localize on the exterior of the hexameric complex (Fig. 2A). The width of the N-ring of the four complexes does not differ much; however, the WM-ATP and MW-ATP complexes appear to be in a contracted conformation that is slightly shortened in height (Fig. 2A). To enable quantitative comparison, we generated quasi-atomic models for the four complexes by flexible fitting of the crystal structure of the MecA-ClpC complex into cryo-EM maps, and the overall fitting is reasonably good (Table 1).
models as feature markers. As shown, protrusions of densities could be attributed to the L1 loops, and there is an apparent difference on the L1 position among the four maps. The L1 loops of the MM-ATP and MW-ATP complexes are in a higher axial position (Fig. 3B), compared with those of the MM-ADP and WM-ATP complexes. This observation indicates that, in analogy to the reported crystal structure of ClpX (18), nucleotides could also modulate the axial position of the D1 loops in ClpC. Notably, ClpX is equivalent to the D2 ring of the MecA-ClpC complex. As to the L3 pore, all four maps display a seemingly occluded L3 pore at low threshold (Figs. 2C and 3). At higher density threshold, the L3 pore loops of the WM-ATP and MW-ATP complexes appear to be more disordered (Fig. 2D). A recent crystallography study on the isolated D2 domain of ClpB (64) also reported a nucleotide-dependent immobilization of L3 loops. Altogether, these data confirm a nucleotide-dependent regulation on the conformations of both the D1 and D2 pore loops in the type II AAA\(^+\) hexamers.

**N Termini of MecA Control the Substrate Access to the Unfoldase**—In the four cryo-EM maps, clusters of densities, scattered or packed, appear above the open chamber of the N-ring (Figs. 2, A and B, and 3). At the N termini of MecA, the substrate could be translocated into the D1 compartment of the MecA-ClpC complex.
form a “specificity filter” that only allows the access of substrates with MecA-specific recognition motif to the lower pore loops on the D1 ring. Consistent with this notion, the CTD of MecA could act as a degradation tag when fused to other unrelated proteins (41). Therefore, it demonstrates that MecA-mediated substrate recognition is in principle dependent on the proximity of the substrate to the unfoldase pore loops.

Visualizing the Translocation of the N Termini of MecA Inside the Unfoldase Chamber—We also observed densities corresponding to the interaction of MecA N termini with the pore loops from the D1 ring (Figs. 2B and 3). This indicates that MecA degradation by the ClpCP complex likely occurs in a cis fashion, which would result in a quick disassembly of the MecA-ClpCP complex itself (41). This property of the MecA-ClpC complex, we believe, is of important physiological relevance, because it enables not only the specific degradation of ComK in a timely manner but also the prompt inactivation of unloaded unfoldase to avoid unnecessary energy consumption.

Judged from the cryo-EM maps, the densities corresponding to the interaction between MecA and the D1 pore loops are stronger in the presence of ATP (Fig. 2B). These observations correlate well with published biochemical data (19, 63) showing that the interaction between the pore loops and the substrate in the ATP-bound state of ClpX is much stronger than that in the ADP-bound state. Importantly, in the maps of the WM-ATP complexes, significant densities appear in the C2 ring (32, 67). By contrast, with the D1 ring as reference, the D2 rings of the cryo-EM structures are in a more compact form than in the crystal structure (Fig. 5A). Further analysis indicates that the compactness of the D2 ring can be further modulated by ATP hydrolysis. The sharpest contrast is between the MW-ATP and WM-ATP complexes. Compared with the WM-ATP complex, the D2 ring of the MW-ATP complex exhibits a significant outward movement for the large subdomains and a rotational movement for the small subdomains (Figs. 4 and 5, B and C).

Consequently, this leads to significant changes on the constellation of the six VGF loops of the D2 large subdomains (Fig. 5, B and C). The VGF loop is conserved among ClpP-targeting HSP100/Clp family proteins and is required for binding to ClpP (24, 65, 66).

Conformational Changes Induced by Nucleotide Binding—Atomic models enable the comparison of cryo-EM structures in a pseudo-quantitative manner. We first compared two atomic models of the MM-ATP and MM-ADP complexes at the NBD ring level, aiming at identification of general interring motions.

With the N-ring as reference for alignment, the D1 ring appears to undergo a noticeable rotational movement between the ATP-bound and ADP-bound states (Fig. 6A). This result indicates that the D1 pore loops are capable of undergoing rotational movement around the pore axis, in addition to the axial movement (Fig. 3), in response to the binding of different nucleotides. A close-up view of a single NBD shows that the D1 small subdomain maintains roughly the same position, whereas both the D1 large subdomain and M domain display an apparent movement in a scale of 5–6 Å (Fig. 6B). In agreement with this observation, the M domain of ClpB was known to change its conformation in response to nucleotide modulation on the D1 ring (32, 67). By contrast, with the D1 ring as reference, the D2 rings of the two complexes differ in another pattern (Fig. 6C). The D2 small subdomains in the MM-ADP complex appear to undergo a rotational movement around the hinge between the large and the small subdomains, whereas the D2 large subdomains remain largely unchanged (Fig. 6D).
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The observations derived from comparison of pseudoatomic models are consistent with those based on analysis of the difference maps (Fig. 4). Although it is impossible to quantitate the inter-subdomain motions at current resolution, our results agree with the general mechanism of nucleotide modulation on the inter-subdomain orientation of the AAA+ proteins (68).

ATP Hydrolysis Induces Dramatic Inter-ring Rotation — Next, the atomic models of the MW-ATP and WM-ATP complexes were similarly compared. The two atomic models were aligned using the N-ring as reference. However, there is no apparent rotation of the D1 ring as a whole (Fig. 6E), although certain degrees of inter-domain motions on the D1 ring could be noticed (Fig. 6F).

When the D1 ring was used as reference, the D2 ring of the WM-ATP complex undergoes a 4° clockwise rotation (top view) compared with the MM-ATP complex (Fig. 7). In sharp contrast, the D2 ring of the MW-ATP complex is rotated anticlockwise by 5° (top view), relative to the MM-ATP complex (Fig. 7), which results in a maximal rotational contrast between the D2 rings of the WM-ATP and MW-ATP complexes (Fig. 6G). Intriguingly, the relative position of the D2 rings from the four samples seems to be in a continuous trajectory (Fig. 7), in an order as MW-ATP, MM-ATP, MM-ADP, and WM-ATP complexes (clockwise, top view). Other than the overall rotation of the D2 ring as a whole, both the large and small subdomains are seen to have apparent displacement (Fig. 6H).

In summary, the conformations of the WM-ATP and MW-ATP complexes appear to be sharply different from those of the MM-ATP and MM-ADP complexes, and pronounced conformational changes, particularly the inter-ring rotations, are only apparent in the samples with active NBDs. This means that the structures of the former two are not a simple linear combination of the latter two, and the saturation of the MM complex with either ATP or ADP is insufficient to induce full scale structural remodeling.

Structural Remodeling of the ClpC Protomers in the Four Atomic Models — To understand the possible mechanism of the conformational signal propagation within a single ClpC subunit, we compared the four models in terms of a single protomer. Because of the apparent central role of the D1 small subdomain in conformational signal transmission, we aligned four ClpC protomers using the D1 small subdomain as reference. Notably, this alignment would sensitize the inter-domain motion within a single protomer and facilitate the tracking of local conformational changes that contribute to the overall structural dynamics described above. As a result, pairwise comparisons of the four models revealed three prominent features.

First, the interface between the NTD of ClpC and the CTD of MecA is relatively rigid, and they often move collectively. For example, compared with the MM-ATP complex, the ClpC-NTD-MecA-CTD subcomplex in the MM-ADP complex flips in an outward direction, resulting in further opening of the N-ring (Fig. 8A and supplemental movie 2). Whereas in the MW-ATP complex, this subcomplex moves as a whole toward the D1 ring (Fig. 8, C and E, and supplemental movies 3 and 4). Together with the dramatic upward movement of the D2 subdomains, this results in a vertical compression of the MW-ATP complex (Fig. 8, C, E, and F, and supplemental movies 3 and 4). The movement at the distal N-ring is apparently mediated by the M domain, which bridges the D1 small subdomain and MecA and also displays a concerted movement (Fig. 8, B–E). This suggests that the M domain is crucial for the signal propagation from the D1 ring to the N-ring.

Second, the interface between the D1 small subdomain and D2 large subdomain substantially reorganizes from one complex to another (Fig. 8 and supplemental movies 2–5). This interface change is dramatized in a morphing movie between the protomers of the MW-ATP and WM-ATP complexes (supplemental movie 5). As shown, a sliding motion between the D2 large subdomain and D1 small subdomain was observed. This suggests that this D1-D2 interface could potentially transmit a motion signal between the D1 and D2 rings. Consistently, the motions on the D1 and D2 subdomains appear to be coupled. When the D1 large subdomain moves up and away from the center, leading to a decrease in the D1 pore diameter, the D2 large subdomain moves up and toward the center, resulting in a contraction of the D2 pore (supplemental movies 2 and 5). Oppositely, when the D1 large subdomain undergoes centriple-
tal movement, the D2 large subdomain rotates away from the center (supplemental movie 6).

Third, both the axial and radial positions of the D1 pore loops are different in the four types of protomers. The D1 pore loop of the MW-ATP protomer is placed at the comparatively highest axial position (Fig. 8, C, E, and F). These observations are in agreement with the analysis based on density maps (Fig. 3), indicating that the nucleotide hydrolysis on the D2 ring could potentially regulate the axial position of D1 pore loops. The L1 loop of the WM-ATP protomer appears to be in an innermost position (Fig. 8, B, D, and F), whereas that of the MM-ADP protomer is the outermost one (Fig. 8, A, D, and E). In fact, neither the D1 nor D2 large subdomain in the four protomers moves strictly in a radial or axial direction. This indicates that both the D1 and D2 loops move in a twisted fashion during an unfolding cycle. These changes on the radial and axial positions of the pore loops, we believe, reflect a general trend of loop movements originating from the relatively rigid motion between the large and small subdomains. This suggests that both the size and axial position of the respective pores could change.

In summary, the comparison of protomers of the four structures reveals that the nucleotide binding and hydrolysis remodel the hexameric complex extensively, including the opening of the N-ring, inter-ring rotations, relative orientation changes between the large and small subdomains within individual NBDs, as well as the consequent changes on the positions of pore loops. These observations demonstrate that the MecA-ClpC complex is a highly dynamic machine.

Proper Configuration at the D1-D2 Interface Is Required for Maximal Unfoldase Activity—Our structural analysis reveals that the large scale inter-ring rotation of the MecA-ClpC com-
plex takes place when active NBDs are present in the complex and indicates there is a transmission of conformational signal at the D1-D2 interface.

To confirm the contribution of inter-ring communication to the overall activity of the hexameric unfoldase, we first tested the role of the linker at the D1-D2 interface of ClpC (Fig. 9). Both the increase and decrease of the linker length have a deleterious effect, significantly lowering the ATPase and unfolding activities of the hexameric complex. Lengthening the linker by five Gly and five Ala residues reduced these activities to 30% of the WT levels. Successive deletions of the linker sequence result in progressively impaired biochemical activities, with the seven-residue deletion 486–492 nearly abolishing both activities. These experiments suggest that the length of the D1-D2 linker in ClpC is critical for its function. Next, point mutations to Ala residue were introduced to six selected positions at the D1-D2 interface. These ClpC variants all display several reduced biochemical activities, with the exception of R528A, which retains 80% activities of WT levels. The most deleterious effect is from the E495A mutation, which is next to the linker sequence. These mutational experiments reveal that the proper configuration at the inter-ring interface is required for the maximal activities of the MecA-ClpC complex, confirming the presence of conformational signal propagation between the two NBD rings.

**DISCUSSION**

**Structural Dynamics of the Type II AAA$^+$ Hexamers**—In this study, we analyzed the cryo-EM maps of four relevant MecA-ClpC complexes in a semi-quantitative manner. Through the structural analysis, we have revealed that, as exemplified by the MecA-ClpC complex, the type II AAA$^+$ machines are extremely versatile. During a functional cycle, all three rings undergo structural remodeling. The N-ring exhibits a flip motion (supplemental movies 5 and 7), which could result in the opening or close of the N-ring. As to the D1 and D2 rings, neither of their subdomains moves simply in a radial or axial direction. Instead, they rotate around the inter-subdomain hinges. The direct output of these nucleotide-dependent local motions is that it realizes corresponding changes on the sizes and axial positions of the successive pores. As a consequence, the pore loops in fact move in a spiral trajectory. This means that, during substrate translocation within the unfoldase chamber, an additional twisting force is applied to the substrate peptide backbone. Such a twisting likely helps improve the unfolding and translocation efficiency, because it would increase the probability of the loop residues to catch the substrate through a longer trajectory of movement, which is reminiscent of nature’s design of a food processor.

Importantly, the motions on individual rings are coupled to each other. When the D1 pore tends to contract (as in the WM-ATP complex), the D2 pore dilates, and vice versa (as in the MW-ATP complex) (supplemental movies 5 and 6). This clearly suggests a motion synchronization of the two rings. Consequently, nucleotide binding and hydrolysis lead to a change in the sizes of the two compartments. The plasticity of the unfoldase chamber reflects an exceptional ability of the type II AAA$^+$ proteins to handle a great variety of substrates. In addition, there is also a rotational movement between the two rings. Together with previous structural observations on other type II AAA$^+$ proteins (30, 31, 38), it appears that the relative change on the stacking angle between two NBD rings during a functional cycle is a common feature for type II AAA$^+$ proteins.

Apart from the inter-ring rotation, we found that nucleotide binding and hydrolysis also modulate the compactness of the D2 ring and lead to the change of the constellation of six ClpP-interacting VGF loops. It was shown that ClpX has a higher affinity for ClpP in the presence of ATPγS, and multiple IGF loops in ClpX (VGF equivalent) are required for functional binding to ClpP (69, 70). Therefore, our observed changes on the spacing between VGF loops might reflect the underlying mechanism of nucleotide regulation on the association of the MecA-ClpC complex with ClpP, in the presence of a symmetry mismatch between the hexameric unfoldase and 7-fold ClpP peptidase.
There are 12 nucleotide-binding sites in a single MecA-ClpC hexamer, forming two stacked AAA\(^+\) rings. In addition to the inter-ring allosteric mechanism, structural dynamics of type II AAA\(^+\) hexamers are also compounded by inter-subunit allosteric regulation (31, 64, 71). Although our structural study could not provide any clue to the inter-subunit conformational signaling due to the symmetry imposition, it has revealed several major motion modes for the type II AAA\(^+\) proteins and could serve as a framework for further biochemical investigations.

**Inter-ring Communication and Allosteric Regulation between the D1 and D2 Rings**—Importantly, our analysis shows that the relative rotation between the D1 and D2 rings in the MM complexes induced solely by ATP or ADP binding is considerably smaller than that observed in the WM or MW complexes in the presence of ATP. As to the WM-ATP and MW-ATP complexes, both were incubated at 37 °C for 40 min before cryo-freezing, which seems to be sufficient for the full conversion of all ATP molecules to ADP, based on the ATPase activity measurements of individual NBD rings (24). Thus, one would expect that only ADP molecules are left in the two complexes. However, the structural data show that the WM-ATP and MW-ATP complexes are in sharply different conformations compared with the MM-ADP complex. A simple explanation is that ADP molecules generated by the active NBDs in the WM-ATP and MW-ATP complexes could not fully replace the bound ATP molecules in the inactive NBDs, resulting in heterogeneous binding states (ATP and ADP) for the two NBD rings. In fact, previous isothermal titrations of the ClpB complex with ATP and ADP revealed that the binding of ATP, but not ADP, to the D2 ring stimulates nucleotide binding to the D1 ring (61). Our results therefore provide direct structural evidence for the presence of allosteric regulation of nucleotide binding to the D1 and D2 rings during a functional cycle of the MecA-ClpC complex.

Our mutational experiments further confirm that the conformational signal is conveyed through the D1-D2 interface. The length of the D1-D2 linker is highly conserved among ClpA, ClpB, and ClpC. As expected, our results show that the changes in the linker length impair both the ATPase and unfolding activities of the MecA-ClpC complex. Shortening the linker apparently would impair the inter-ring rotation. Also, lengthening the linker would jam the D1-D2 interface, producing similar effects on the unfoldase activities. Point mutations on a few positions at the D1-D2 interface, also affect the overall activities, indicating that specific atomic recognition between the D1 and D2 is required for the maximal activities. Notably, biochemical studies on similar WM and MW mutants of ClpB (72, 73), as well as on other different mutants of type II AAA\(^+\) unfoldases, such as ClpB (74), HSP104 (75, 76), and p97 (71), have also suggested the presence of inter-ring communication. Therefore, there must be a cooperativity of the two AAA\(^+\) rings of the general type II unfoldases.

Altogether, it suggests that the two rings likely work in a concerted, cyclic manner, probably to prevent the backward slippage of the substrate. As one ring finishes a single round of “pulling,” the other ring is just ready to “hold” the substrate.
According to this speculaton, a smooth and effective handover of substrates between the two rings could be achieved. With an emphasis on the inter-ringer allostery control, we generated a movie morphing between the cryo-EM maps of the WM-ATP and WM-ATP complexes (supplemental movie 7), which gives a simplified representation of the dynamic cycle of the MecA-ClpC molecular machine.

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**Cryo-EM Study of the MecA-ClpC Complex**