Helix Unfolding/Refolding Characterizes the Functional Dynamics of Staphylococcus aureus Clp Protease*

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Background: The molecular mechanism of ClpP dynamic switching between different conformations is poorly understood.

Results: MD simulations describe the molecular pathway of the transition between three conformations of SaClpP.

Conclusion: Helix unfolding/refolding characterizes the functional dynamics and mechanism of ClpP.

Significance: This study provides molecular insights into the dynamics and mechanism of ClpP in general.

The ATP-dependent Clp protease (ClpP) plays an essential role not only in the control of protein quality but also in the regulation of bacterial pathogen virulence, making it an attractive target for antibacterial treatment. We have previously determined the crystal structures of Staphylococcus aureus ClpP (SaClpP) in two different states, extended and compressed. To investigate the dynamic switching of ClpP between these states, we performed a series of molecular dynamics simulations. During the structural transition, the long and straight helix E in the extended SaClpP monomer underwent an unfolding/refolding process, resulting in a kinked helix very similar to that in the compressed monomer. As a stable intermediate in the molecular dynamics simulation, the compact state was suggested and subsequently identified in x-ray crystallographic experiment. Our combined studies also determined that Ala140 acted as a “hinge” during the transition between the extended and compressed states, and Glu37 was essential for stabilizing the compressed state. Overall, this study provides molecular insights into the dynamics and mechanism of the functional conformation changes of SaClpP. Given the highly conserved sequences of ClpP proteins among different species, these findings potentially reflect a switching mechanism for the dynamic process shared in the whole ClpP family in general and thus aid in better understand the principles of Clp protease assembly and function.

The number of bacterial strains resistant to antimicrobial agents is continuously increasing, thus warranting the rapid development of new drugs with novel mechanisms of killing bacterial cells (1, 2). Proteases are a class of promising bacterial targets that play an important role in protein quality control by degrading short lived regulatory proteins as well as misfolded or damaged proteins, thereby maintaining cellular homeostasis (3, 4). The ATP-dependent Clp protease (ClpP)6 is a highly conserved serine protease present in bacteria and eukaryotes (5). The proteolytic core of ClpP needs to be associated with ATPases, such as the chaperones ClpX and ClpA, to perform its functions (6). ClpP plays a crucial role in the survival and virulence of pathogenic bacteria during host infection (7–10). Frees et al. (8) found that clpP deletion (ΔclpP) in Staphylococcus aureus resulted in strongly decreased extracellular virulence. The ΔclpP mutant also led to up-regulation of several transcriptional repressors of virulence genes (e.g. SarA family) and down-regulation of the virulence inducing the global regulatory agr quorum-sensing system (9). Recently, ClpP has been extensively studied as a target for both antibacterial and antivirulence interventions (11–13). Acyldepsipeptides (14) have been identified as a new class of antibiotics that target ClpP to dysfunctional degrad cell division protein like FtsZ (11, 15, 16).

Crystal structures of ClpP from several organisms have been determined, and they show that two heptameric rings enclose a large chamber containing 14 proteolytic active sites. The structure of a ClpP monomer can be divided into three parts: the flexible axial loop, the rigid head domain, and the dynamic handle domain (Fig. 1A) (17). Based on the oligomer assembly and

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Dynamic Switching of S. aureus ClpP

conformation of the handle domain, these structures can be classified into three distinct structural states: extended, compact, and compressed (Fig. 1). The extended state has been observed in the crystal structures of ClpP from Escherichia coli (16–19), Helicobacter pylori (20), Bacillus subtilis (15, 21), Homo sapiens mitochondria (22), and S. aureus (23–25) (Fig. 1A). In this state, the handle domains from opposing seven-membered rings are well ordered and interlocked to keep the ClpP equatorial surface continuous. Helix E of the handle domain adopts a straight conformation and extends from Ala133 to Thr158, and the catalytic triads are well organized in a catalytically active configuration. The compact state is found in the ClpP from E. coli (26), Streptococcus pneumonia (27), Mycobacterium tuberculosis (28), and Plasmodium falciparum (29) (Fig. 1B). In this state, residues 125–137 of the handle domain are unstructured, and the first two turns of helix E are unfolded such that the helix now runs from Ala140 to Thr158. The compressed state has recently been structurally characterized in S. aureus ClpP (SaClpP) (Fig. 1C) (23, 24). In this state, the long helix E is interrupted by a kink centered on residue Lys145, leading to two short helices (Ala133–Leu140 and Thr146–Thr158). The 126–145 segment in the handle domain, which undergoes large conformational changes, is designated the “dynamic region” of ClpP hereinafter. The catalytic triads are disorganized in both the compressed and compact states, indicating that these two states might be catalytically inactive to cleavage substrates. The superimposition of three single monomers extracted from the three states shows that the conformations of the head domains are almost identical in these structures, whereas the handle domain undergoes dramatic conformational changes (Fig. 1D). It has been previously proposed that SaClpP switches dynamically between the extended and compressed states to perform its catalytic function (23, 26). In addition, these two conformations represent the two states for substrate recruitment and product release, respectively. However, to date, crystal structures for all three states of ClpP have not been solved from a specific species. In addition, the mechanism and pathway of this dynamic transition as well as the role of the compact state during this transition remain largely unknown. Moreover, very little is known about the mechanism by which the compressed state returns to the extended state.

In this study, we conducted a mechanistic investigation on the dynamic transition of SaClpP using a combination of long term molecular dynamics (MD) simulations and X-ray structural analysis. The MD simulations showed that the long and straight helix E of the handle domain in the extended state underwent an unfolding/refolding process during the conformational transition, resulting in a kinked conformation very similar to what was observed in the compressed state. The compact state is suggested by the MD simulations as a stable intermediate during this process that is characterized by the crystal structure of the compact SaClpP. The two turns of helix E in the N-terminal domain (up to Ala139) of this state are unfolded, validating Ala140 as a “hinge” during the transition between the extended and compressed states. Along with our previously determined crystal structures of wild-type SaClpP in the extended and compressed states (23), the present study describes, for the first time, the extended, compact, and compressed forms of ClpP from the same organism (Fig. 1). The mechanism by which the compressed state returns to the extended state was also investigated in this study. Residue Glu137 has been observed to play a crucial role in stabilizing compressed SaClpP, as substantiated by MD simulations and the crystal structure of SaClpP E137A mutant obtained in this study. Taken together, our simulation results and experimental observations present a schematic mechanism underlying the conformational transition pathway between the extended and compressed states and through the compact state of SaClpP.

EXPERIMENTAL PROCEDURES

Molecular Dynamics Simulations—MD simulations were performed in the Gromacs 4.5.3 package (30) by using the NPT ensemble. The missing N-terminal residues of some monomers were added using PyMOL (31). The AMBER03 force field (32) with explicit TIP3P water (33) were used to run MD simulations. The water box was 10 Å away from the protein on all sides (i.e. the starting structure had a 20-Å interval between periodic images). All of the bonds with hydrogen atoms (e.g. C–H and O–H) were constrained using the linear constraint solver algorithm (34), whereas the formation and destruction of hydrogen bonds (e.g. O–H–N) were carefully monitored. Realistic ionic strength is important for the compactness and conformational motion of proteins (35–39), and Na+ and Cl− ions were added to neutralize the simulation systems under simulated physiological conditions (for more details, see supplemental Table 1). The concentration of NaCl in the simulation system is 0.15 M. Long range electrostatic interactions were treated using the particle mesh Ewald method (40). Periodic boundary conditions were applied to avoid edge effects in all calculations. The temperature was kept constant at 300 K by separately coupling the water, ions, and protein in a thermal bath using the Berendsen thermostat method (41) with a coupling time of 1 ps. Berendsen pressure coupling (41) was used for the equilibration of the systems. A cut-off distance of 10 Å was applied for the Lennard-Jones interactions. Before the MD simulation run, the systems were subjected to energy minimizations using the steepest descent algorithm (42). The systems were heated gradually from 0 to 300 K. Finally, conventional MD was performed, with coordinates saved every 10 ps throughout the entire process.

Principal Component Analysis (PCA)—PCA (43) was carried out to address the collective motions of SaClpP using the positional covariance matrix C of the atomic coordinates and its eigenvectors. The elements of the positional covariance matrix C are defined as follows,

\[ C_{ij} = \langle(x_i - \langle x_i \rangle)(x_j - \langle x_j \rangle) \rangle \]

(Eq. 1)

where \( x_i \) is a Cartesian coordinate of the \( i \)th Cα atom, \( N \) is the number of the Cα atoms considered, and \( \langle x_i \rangle \) represents the time average over all of the configurations obtained in the simulation. The eigenvectors of the covariance matrix, \( V_k \), obtained by solving \( V_k C V_k = \lambda_k \), represent a set of \( 3N \)-dimensional directions or principal modes along which the fluctuations observed in the simulation are uncoupled with respect to one another and can be analyzed separately.
Dynamic Switching of S. aureus ClpP

Free Energy Landscape (FEL) Analysis—The FEL for the conformational change of a protein or a protein complex can be obtained using an appropriate conformational sampling method. In this study, conformations produced by MD simulations were used for FEL analysis. A two-dimensional representation of FEL was obtained by selecting PC1 and PC2 as the coordinates could be obtained using the following equation (44–46),

\[ G(q_1, q_2) = -k_B T \ln P(q_1, q_2) \]  

(Eq. 2)

where \( k_B \) is the Boltzmann constant, \( T \) is the simulation temperature, and \( P(q_1, q_2) \) is the normalized joint probability distribution. The energy surface obtained from the raw data were further smoothed using the kernel density smooth method. The energy surface obtained from the raw data were collected at the Shanghai Synchrotron Radiation Facility Beamline 17U. All x-ray data were processed using the HKL2000 program suite (47) and converted to structural amplitudes using the CCP4 program (48). The structures were phased by molecular replacement in Phaser using a previously published SaClpP monomer as the search model (Protein Data Bank code 3ST9). The computational refinement of the model was carried out with the program REFMAC5 in the CCP4 suite (49). The data collection and refinement parameter statistics for the two structures are summarized in Table 1. Molecular graphic figures were prepared with PyMOL (30).

Enzymatic Activity Assay—The activity assay was carried out using the model fluorescence-labeled substrate N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (Suc-LY-AMC) (Shanghai GL Biochem Ltd.). Wild-type SaClpP and the E137A mutant (2 mg/ml) were prepared in a buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, and 50% glycerol) for stock. For Michaelis-Menten kinetics, Suc-LY-AMC stocks (in dimethyl sulfoxide, 100 mM) were prediluted with a reaction buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, and 50% glycerol) to different concentrations (100, 200, 400, 600, 800, and 1000 μM). SaClpP protein (10 μl, 2 mg/ml) mixed with 40 μl of reaction buffer was first added to a black COSTAR 96-well flat bottom plate (Corning Inc.). Then 50 μl of Suc-LY-AMC pre-diluted buffer was added to a final volume of 100 μl. The test reaction was measured in triplicate at 37 °C for 15 cycles every 2 min using a 2104 Envision Multilabeled Reader (PerkinElmer Life Sciences) at 340-nm excitation and 450-nm emission. All data were processed using GraphPad Prism 5 software.

RESULTS

Unfolding/Refolding Process of Helix E in Monomeric SaClpP—Generally, helix E adopts dramatically distinct conformations in the extended, compact, and compressed states (Fig. 1). Based

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**Table 1**

| Data collection and refinement statistics | Compacted SaClpP (PDB code 4EMM) | Extended SaClpP E137A (PDB code 4EMP) |
|------------------------------------------|-----------------------------------|----------------------------------------|
| Data collection                          |                                   |                                        |
| Space group                              | P2                                | C2                                     |
| Cell dimensions                          |                                   |                                        |
| a, b, c (Å)                              | 96.38, 170.05, 96.33               | 168.73, 96.06, 192.43                   |
| a, b, c (degrees)                        | 90, 102.9, 90                      | 90, 91.2, 90                           |
| Resolution (Å)                           | 50–2.40 (2.49–2.40)               | 50–2.60 (2.69–2.60)                    |
| No. of observations                      | 419,000 (44,372)                   | 274,406 (27,150)                       |
| No. of unique observations              | 112,798 (11,677)                   | 88,480 (9050)                          |
| Rsym                                     | 0.106 (0.470)                      | 0.129 (0.855)                          |
| I/mI                                      | 9.6 (3.9)                          | 8.0 (1.1)                              |
| Completeness (%)                         | 96 (100)                           | 95.5 (98.6)                            |
| Redundance ( Å)                          | 3.7 (3.8)                          | 3.1 (3.0)                              |

**Data refinement**

| Resolution (Å)                           | 30–2.40 (2.46–2.40)               | 30–2.70 (2.77–2.70)                    |
| No. of reflections                       | 107,042 (5664)                    | 76,466 (5735)                          |
| Rwork/Rfree                             | 24.0/28.6                          | 23.8/29.0                             |
| No. of atoms                             |                                   |                                        |
| Protein                                  | 18,694                             | 20,005                                 |
| Water                                    | 32                                 | 23                                     |
| Mean B value (Å²)                        | 51.5                               | 60.4                                   |
| r.m.s deviations                         |                                   |                                        |
| Bond lengths (Å)                         | 0.011                              | 0.010                                  |
| Bond angles (degrees)                    | 1.282                              | 1.248                                  |
| Ramachandran plot*                       | 97.9                               | 96.9                                   |
| Allowed (%)                              | 2.1                                | 2.9                                    |
| Disallowed (%)                           | 0.1                                |                                        |

* The shell with the highest resolution is shown in parentheses.

* Calculated in CCP4 suite using Procheck.
on a 200-ns MD simulation of one monomer extracted from the extended state of SaClpP (ClpPex/mono), we have previously proposed that the dynamic region of the handle domain is very flexible and that the kink is spontaneous (23). In the present study, to further investigate the particular conformational changes in the ClpP monomer, we prolonged the simulation of our previous work by performing a 3000-ns MD simulation on ClpPex/mono. We monitored the r.m.s. deviation (RMSD) values of Cα atoms from the head domain and handle domain relative to the initial x-ray crystal structure along the entire MD trajectory, respectively (Fig. 2A). The RMSD profile indicated that handle domain underwent dramatic conformational changes during the simulation, whereas the head domain was much more stable, which is consistent with the crystal structure (Fig. 1D, bottom). The r.m.s. fluctuations were also monitored along the MD trajectory of ClpPex/mono, with the values for individual residues in ClpPex/mono indicating that large fluctuations occurred in the dynamic region, thus validating that this segment is very flexible (data not shown).

Next, to examine the detailed conformational transition of the handle domain (residues 126–158), we calculated the transformation of the secondary structure of ClpPex/mono along the trajectory of simulation by DSSP (50) (Fig. 2B). The profile of the secondary structure transformation and snapshots of the handle domain extracted from the MD trajectory revealed that the helical structure of residues 146–158 was stable during the simulation, which is consistent with the experimental observations (Fig. 1D). However, the N-terminal part of helix E (residues 133–145) underwent a helical unfolding/refolding process. In the initial stage (0–1000 ns), the first two turns of the helix (residues 133–140) unfolded and adopted turn structures (see the 500-ns snapshot in Fig. 2C). Subsequently (1000–2000 ns), these residues gradually readopted some α-helical character (see the 1000-ns, 1500-ns, and 2000-ns snapshots in Fig. 2C), and the degree of kinking in the 133–158 segment increased (Fig. 2C). Finally (2000–3000 ns), the 133–140 segment stabilized in the helical structure (see the 2000-ns, 2300-ns, 2700-ns, and 3000-ns snapshots in Fig. 2C). The snapshot at 3000 ns shows that the long helix E was consequently broken into two shorter helices at the position of residue Ala140. During the entire 3000-ns simulation, the helical structure of residues 141–145 also showed some evidence of unfolding (Fig. 2B). This result suggests that the unfolding/refolding conformational changes might eventually propagate into this part of helix E as well. Although the compressed state, in which helix E was broken at Lys145, was not achieved during the simulation, a remarkable tendency toward this event was observed (Fig. 2B). All of these results showed that ClpPex/mono might spontaneously undergo a conformational transition from the extended state to a conformation very similar to that of the compressed state.

Conformational Changes of Oligomeric SaClpP—Helix E of ClpPex/mono is very flexible, so what keeps it in the straight conformation? We previously proposed that there is an Arg171–Asp170 network at the equator of the tetradecameric cylinder, which is composed of extensive hydrogen bonding and salt bridge interactions mediated through Arg171 and Asp170 from opposing subunits, playing a crucial role in the stability of the extended state of SaClpP (Fig. 3A) (23). Here, 400-ns MD simulations were performed on two systems, respectively: wild-

![FIGURE 1. Three states observed in ClpP. A, structure of SaClpP (Protein Data Bank code 3STA) showing the ClpP tetradecamer in the extended state. The axial N-terminal loops are shown in green, the head domain is shown in cyan, and the handle domain is shown in magenta. B, structure of SaClpP (Protein Data Bank code 4EMM) showing ClpP in the compact state. The color coding used is the same as that in A. The broken line in magenta represents the disordered part of the handle domain (bottom). C, structure of SaClpP (Protein Data Bank code 3ST9) showing the enzyme in the compressed state. D, alignment of three states. The extended, compact, and compressed monomers are shown in pink, purple, and magenta, respectively.](image-url)
type SaClpP extended tetradecamer (ClpP\textsuperscript{ex}) and its D170A/R171A mutation (ClpP\textsuperscript{ex/D170A/R171A}). We monitored the RMSD values of protein C\textsubscript{H9251} atoms on the handle domain and head domain relative to the initial x-ray crystal structure along the MD trajectories, respectively. For the wild-type ClpP\textsuperscript{ex}, both the handle domain and the head domain show similar fluctuations (Fig. 3\textit{B}, top). However, for ClpP\textsuperscript{ex/D170A/R171A}, the RMSD values of the handle domain are much higher than those of the head domain (Fig. 3\textit{B}, bottom). The results suggest that Asp\textsubscript{170}-Arg\textsubscript{171} network is crucial for the stability of extended SaClpP. Because the network disappears in one heptameric ring of the extended SaClpP (ClpP\textsuperscript{ex/hepta}), it cannot be stabilized in the extended state.

Next, to track the conformational transition of oligomeric SaClpP starting from the extended state, we carried out a 1500-ns MD simulation on ClpP\textsuperscript{ex/hepta}. The RMSD profile and the r.m.s. fluctuation values of each residue confirm that large fluctuations of residues occurred in the dynamic region (data not shown). To identify the most significant motions of ClpP\textsuperscript{ex/hepta}, we performed PCA (43) using the MD trajectory. This analysis considers each motion as the variance of atomic fluctuations around the average structure. The first mode (supplemental Movie 1) represents the largest atomic fluctuation in the sampled conformations. As shown in Fig. 3, \textit{C} and \textit{D}, the first component (PC1) consisted of several motions. First, helix E acquired a kinked conformation, resulting in a reduction in ClpP height (excluding N-terminal loops) (Fig. 3\textit{E}). Second, residues 19–125 in the head domain moved downward and inward to the cavity of ClpP, leading to a reduction in the N-terminal radius of ClpP (Fig. 3\textit{F}, top). Finally, helix F moved out-
ward, resulting in an increase in radius of the whole structure (Fig. 3F, bottom). As shown in Fig. 3G, the overall dimensions of one heptameric ring from the extended SaClpP (ClpP<sup>ex/hepta</sup>) were 50 Å in height (excluding N-terminal loops) and 50 Å in radius. The axial pore surrounded by Asp<sup>170</sup>-Arg<sup>171</sup> was 12 Å in radius. The corresponding dimensions of one heptameric ring from the compressed SaClpP were 37, 54, and 10 Å (Fig. 3G). These motions led to the compression of ClpP, which coincides with the crystal structures of SaClpP, suggesting that the extended ClpP shows a remarkable tendency toward the compressed state without the Asp<sup>170</sup>-Arg<sup>171</sup> network. During the 1500-ns MD simulation, segment 133–140 showed helical unfolding (data not shown), indicating that the helix unfolding/refolding process was present in the oligomeric state as well.

The Compact State Represents an Intermediate during the Transition—During the conformational transition of the SaClpP monomer from the extended state to the compressed state, an intermediate conformation in which helix E was broken at Ala<sup>140</sup> (see the 2000-ns, 2300-ns, 2700-ns, and 3000-ns snapshots in Fig. 2C) was observed. Based on the results of MD simulation and PCA, we constructed a rough energy landscape for the conformational transition of ClpP<sup>ex/mono</sup> projected onto the first two principal components, PC1 and PC2, which accounted for 52.7 and 10.6% of the overall motions, respec-
tively (Fig. 4A). PC1 reflected the most dominant motion mode of ClpP$^{\text{ex/mono}}$. Reaction coordinates were defined according to PC1 and PC2 obtained from PCA. The energy landscape consisted of several deep wells, reflecting the transition pathway of the structural compression. Every frame of the structure generated by MD simulation corresponds to two coordinates (PC1 and PC2), and conformations of each well were selected based on the corresponding coordinates. The first deep well corresponded to the extended monomer, which served as the starting structure. Concomitant with conformational changes, ClpP$^{\text{ex/mono}}$ climbed up along the wall and fell into the second minimum. By mapping conformations to this deep well, we found that helix E of the corresponding structure was broken at Ala$^{140}$ (Fig. 4B). Afterward, ClpP$^{\text{ex/mono}}$ moved to the bottom of the third well to achieve a state very similar to that of the compressed ClpP (Fig. 4C). The binding angles between the two helices (134–139 and 141–158) of the second and third minima are 100° and 80°, suggesting the structure of ClpP$^{\text{ex/mono}}$ became more “compacted” during transition. The compressed state was not fully achieved during the simulation; the third minimum (the compressed-like state) might be an intermediate between the second minimum structure and the fully compressed state. Thus, the energy landscape, together with the results of the secondary structure transformation, suggests the presence of an intermediate state during the transition between the extended and compressed states. This intermediate state, wherein helix E was broken at Ala$^{140}$, plays an important role in the conformational transition of this helix.

We determined a crystal structure of wild-type SaClpP at 2.40 Å resolution (for data collection and structure refinement statistics, see Table 1) in which the segment between residues 127 and 138 was disordered in all monomers (Fig. 1B). This structure is similar to the compact state observed in the ClpP structures of S. pneumoniae (A153P) (27), P. falciparum (29), and E. coli (26) (Fig. 3B). Based on the energy landscape of the ClpP$^{\text{ex/mono}}$ conformational transition, we captured an intermediate conformation that corresponded to the second well. The superposition of the second local energy minimum structure with the crystal structure of the compact SaClpP yielded only a small RMSD value (1.13 ± 0.10 Å) averaged over the 14 monomers in the compact SaClpP. Consistent with the position where helix E in the second energy minimum state

**TABLE 2**

Contribution of top 10 PCs generated by PCA

| PC  | Contribution |
|-----|--------------|
| PC1 | 52.7         |
| PC2 | 10.6         |
| PC3 | 7.22         |
| PC4 | 4.33         |
| PC5 | 3.42         |
| PC6 | 2.03         |
| PC7 | 1.79         |
| PC8 | 1.66         |
| PC9 | 1.29         |
| PC10| 0.92         |

**FIGURE 4.** Intermediate compact state during the transition between the extended and compressed states. A, top, energy landscape for the conformational transition of ClpP$^{\text{ex/mono}}$. Reaction coordinates were defined according to PC1 and PC2 obtained from PCA. Bottom, typical conformations of local energy minima. B, structural superimposition of compact states from several organisms and the second energy minimum structure of the energy landscape. C, structural superimposition of compressed state and the third energy minimum structure of the energy landscape. D, hydrolysis of the fluorogenic peptide Suc-LY-AMC by wild-type SaClpP and two mutants (A140G and A140P). Kinetic constants were determined with the GraphPad Prism 5 software by plotting enzyme velocity against substrate concentration. Error bars (S.E.) are shown based on three independent repeats. RFU, relative fluorescence units.
formed kinks, helix E in the crystal structure of the compact SaClpP was also disordered in the N-terminal side of Ala140 (Fig. 4B). The mutation of Ala140 to glycine or proline completely abolished the activity of SaClpP for efficient degradation of the short model peptide Suc-LY-AMC in vitro, indicating that Ala140 in SaClpP is essential for the stability of the compact state and thus plays an important role in the conformational transition and degradation activity of ClpP (Fig. 4C). Consistent with this proposal, the mutation to cysteine (26) or proline (27) of Ala153 in E. coli ClpP, corresponding to Ala140 of SaClpP, reduced the activity partially or completely. Accordingly, we conclude that the compact conformation represents a stable intermediate during the transition between the extended and compressed states. Particularly, Ala140 acts as the hinge in the unfolding/refolding process of helix E in the handle domain.

Glu137 Plays an Important Role in Stabilizing the Compressed State—The driving force that triggers the conformational transition back from the compressed state to the extended state remains unclear. In our previously solved structure of the compressed SaClpP, Glu137 is shown in a stick representation. C, distance of atom OE1 in Glu137 and OD1 in Asp38 versus simulation time. D, overall superimposition of the two rings from the E137A mutant (slate) and wild-type extended SaClpP (pink) is presented in cylindrical schematics. The superimposition was carried out using PyMOL and yielded a small RMSD (0.22 Å for the tetradecamer), indicating high similarity between these structures. E, secondary structures as a function of time for ClpP3000/mono and ClpP3000/E137A in trajectories as calculated using DSSP. Top, profile of secondary structure transformation of ClpP3000/mono. Bottom, profile of secondary structure transformation of ClpP3000/E137A.

To test whether the mutation of Glu137 could destroy these interactions and thus lead to the instability of the compressed state, we determined the crystal structure of the E137A mutant of SaClpP. The structure was refined at 2.60 Å resolution (Table 1). This mutant adopted the extended conformation under conditions similar to those of crystallized SaClpP in the compressed state. Superposition of the structures of the wild-type extended SaClpP and the E137A mutant yielded very small Cα RMSD values, 0.18 Å for the monomer and 0.22 Å for the tetradecamer, which further reveals the high similarity between these two structures (Fig. 5D). In addition, the E137A mutant of SaClpP loses peptidolytic activity, revealing that the Glu137 residue is important for the degradation function of SaClpP (25).
Taken together, this structural character and biochemistry result indicate the essential role of Glu$^{137}$ in keeping the helix kinked in the compressed state (Fig. 5D) and suggest that the compressed state should be an essential factor during the functional cycle of SaClpP.

Next, to investigate the importance of Glu$^{137}$ for the compressed state, we performed 200-ns MD simulations from the monomer of compressed SaClpP (ClpP$^{cpr/mono}$) and its E137A mutant (ClpP$^{cpr/E137A}$), respectively. The profile of the secondary structure transformation shows that the handle domain (especially the dynamic region) of ClpP$^{cpr/mono}$ is more stable than that of ClpP$^{cpr/E137A}$, again revealing that E137 is important for stability of the compressed state (Fig. 5E).

**DISCUSSION**

The conformational transitions of ClpP are closely related to its functional cycle (23, 26). However, they are poorly understood due to the complexity of the whole system. Herein, we report the crystal structure of wild-type SaClpP in the compact state. Along with the extended and compressed states, the structures of which we have determined previously (23), this study describes, for the first time, the three distinct states from the same organism (Fig. 1 and 6). A series of MD simulations were performed to gain mechanistic insights into the dynamics of ClpP at the atomic level. Our MD simulation results and crystal structures suggest that SaClpP switches dynamically between the extended and compressed states, and the compact state represents an intermediate during the transition. Such conformational changes were also observed in other ClpP structures (23, 24, 26, 52). Cross-linked *E. coli* ClpP, which is stuck in the compact state, shows no detectable peptidolytic activity (26), suggesting that the conformational transition is necessary for ClpP function. The dynamic conformational changes are key properties of the ClpP functional cycle, which consists of the postdegradation and recovery steps (Fig. 6).

The extended state of SaClpP is stabilized by an Asp$^{170}$-Arg$^{171}$ network at the equator of the tetradecameric cylinder, which is composed of extensive hydrogen-bonding and salt bridge interactions mediated through Asp$^{170}$ and Arg$^{171}$ from opposing subunits (23, 25, 27, 53) (Fig. 3A). During the postdegradation step, after degradation of substrate peptides, accumulation and release of digested fragments from the active site may induce a rearrangement of the catalytic triad, thereby leading to the destruction of the nearby Asp$^{170}$-Arg$^{171}$ contact network (Fig. 3A). As a result, the extended ClpP transforms into the compressed state, resulting in the formation of equatorial pores, through which digested peptides could be released from the catalytic chamber (27, 54). The conformational transition of helix E involves an unfolding/refolding process, during which the transformation of the secondary structure is gradually propagated along the long helix (Fig. 2). The compact state acts...
as an intermediate conformation, which was captured here by both MD simulation and crystal structural analysis (Fig. 4). In the compact state, helix E becomes disordered in the N terminus of Ala140 (Figs. 1B and 4B), suggesting that this residue acts as a hinge in the conformational changes of helix E. The A140G and A140P mutants in SaClpP fail to degrade peptides in vitro (Fig. 4D), and the mutation to cysteine (26) or proline (27) of Ala153 in E. coli ClpP, corresponding to Ala140 in SaClpP, causes reduced or complete loss of activity, indicating an important role of Ala140 in the conformational transition and degradation activity of SaClpP. Using the energy landscape constructed based on the PCA results of the MD simulation of the SaClpP extended monomer, we observed three energy minimum states. The conformations in the first and second minima agree well with the crystal structures of the extended and compact SaClpP, respectively (Fig. 4A). Although the 3000-ns MD simulation did not reach the fully compressed state of SaClpP, the energy minimum structure in the third well was very close to the compressed state (Fig. 4C). Moreover, change of protonation state of Asp38/Glu137 during transition is propitious to achievement of the compressed state.

During the recovery step, the compressed state undergoes a conformational transition back to the extended state. The key residue Glu137, which is located near the N terminus of helix E, turns out to be essential for the stability of the compressed state. It acts as an anchor between helix E and the head domain through a network of hydrogen binding, thereby keeping helix E in a kinked conformation (Fig. 5A). The crystal structure of SaClpP E137A, which adopts an extended state under conditions similar to those used for crystallizing the compressed SaClpP, further suggests that Glu137 is crucial for the compressed SaClpP (Fig. 5D). Due to loss of the "anchor," SaClpP cannot be steady in the compressed state during the transition. It is perhaps the reason why E137A mutant of SaClpP loses activity (25). Taken together, these results indicate that the compressed state is crucial for the functional cycle of SaClpP. The 200-ns MD simulations also reveal that Glu137 is important for stability of the compressed state (Fig. 5E).

These simulations focus on not only the E helix unfolding/folding but also the whole oligomeric SaClpP. Although the structural change of the E helix is bigger than that of the head domain, the head domain still can move at a comparably large dimension as a whole subunit or intermonomer among the heptamer. Indeed, many other parts also take part in large conformational changes (Fig. 3). The functional dynamics of SaClpP features the unfolding/folding processes of handle helix E; however, it still remains unclear which part is most important for governing this motion.

In conclusion, our study provides new insights into the pathway and mechanism of the dynamic switching of S. aureus ClpP. The MD simulation results, along with x-ray structural and biochemical analysis, suggest a model in which SaClpP switches dynamically between the extended and compressed states to perform its function, and the compact state represents an intermediate during this transition. Our results also highlight the molecular pathway of SaClpP transition between these three conformational states. Given the highly conserved amino acid sequences and similar protein foldings of ClpP from different species, the detailed dynamic switching mechanism of SaClpP that we herein propose provides a solid framework in general for understanding the molecular mechanism of the ClpP family of cylindrical proteases.

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Dynamic Switching of S. aureus ClpP

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