STRUCTURAL SWITCHING OF *STAPHYLOCOCCUS AUREUS* CLPP: A KEY TO UNDERSTANDING PROTEASE DYNAMICS

Jie Zhang1, Fei Ye1, Lefu Lan, Hualiang Jiang, Cheng Luo2, Cai-Guang Yang3

From the State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China

Running head: Structural dynamics of *S. aureus* ClpP

ATP-dependent Clp protease (ClpP) is an attractive new target for the development of anti-infective agents. The ClpP protease consists of two heptameric rings that enclose a large chamber containing fourteen proteolytic active sites. Recent studies indicate that ClpP likely undergoes conformational switching between an extended and degradatively active state required for substrate proteolysis, and a compacted and catalytically inactive state allowing product release. Here, we present the wild-type ClpP structures in two distinct states from *Staphylococcus aureus* (*Sa*ClpP). One structure is very similar to those solved ClpP structures in the extended states. The other is strikingly different from both the extended and the compacted state as observed in ClpP from other species; the handle domain of this structure kinks to take on a compressed conformation. Structural analysis and molecular dynamic simulations show that the handle domain predominantly controls the way in which degradation products exit the chamber through dynamic conformational switching from the extended state to the compressed state. Given the highly conserved sequences among ClpP from different species, this compressed conformation is unexpected and novel, which is potentially valuable for understanding the enzymatic dynamics and the acting mechanisms of ClpP.

Clp proteases such as ClpXP and ClpAP, which catalyze the unfolding and degradation of specific proteins, play a critical role in various processes that regulate cellular functions via proteolysis in both prokaryotes and eukaryotes (1-4). This system has been studied in detail in *Escherichia coli* (5-7), but little is known regarding the specific biological function of mitochondrial ClpP in humans (8). The proteolysis core of ClpP is formed by two heptameric rings of the proteolytic subunit (9). The ATPase specific factor, such as ClpA, ClpC, ClpX or ClpY, is attached to the proteolytic core and determines substrate specificity, allowing suitable substrate to enter the proteolytic chamber (10). Recently, growing evidence has suggested that the ClpP plays a crucial role in the survival and virulence of pathogens including *Staphylococcus aureus* during host infection and thus serves as an attractive new target for anti-infective agents (11-14).

Acyldepsipeptides (ADEP) have been identified as a new class of antibiotics that target ClpP (15).

The crystal structures of ClpP proteins from several different organisms have been experimentally determined. Based on the mode of conformational organization, the ClpP structures are classified into two distinct groups that represent functional active and inactive forms, which are referred to as the extended and compacted states, respectively. In the extended ClpP structures observed from *E. coli* (9,16-18), *Helicobacter pylori* (19), *Bacillus subtilis* (20), and *Homo sapiens* mitochondria (21), the fourteen handle helices are well ordered to hold the double helptameric rings interlocking, thus keeping surface of the ClpP equator continuous. The active site residues in this state are generally organized in catalytically active positions. However, the handle helices are typically unstructured and very few of them could be fully traced in electron density maps in the compacted structures solved in *E. coli* (22), *Streptococcus pneumoniae* (23), *Plasmodium falciparum* (24), and *Mycobacterium tuberculosis* (25). Furthermore, in compacted states, the catalytic triads are disorganized when compared to those in extended states. Although both the extended and compacted structures were solved for *EcClpP*, the latter was crystallized from an engineered protein by using disulphide cross-linking (22). To date, no firm structural data exists that simultaneously shows these two states are accessible to a given wild-type ClpP from a specific species.

Solved ClpP crystal structures have provided valuable information for understanding the process by which a substrate peptide enters a
cylindrical chamber and is recognized in the active site for degradation (17,19). However, how the degraded polypeptides are released from the ClpP proteolytic chamber still remains largely controversial. Investigators have made many efforts to find structural evidence that would elucidate the molecular mechanisms of peptide fragments release. Two major models have been put forth. One model proposes that degraded oligopeptide products exit the catalytic chamber by passive diffusion through the same axial pores that allow the entry of unfolded proteins (26,27). This proposal is problematic, however, because ATPase binds to ClpP simultaneously at both ends, which interrupts product release (28). The other model proposes that the degraded peptides exit the catalytic chamber through side pores generated transiently by the dynamic conformational changes of the handle regions. Studies of X-ray structural analysis of an A153P mutation of SpClpP (23) and quantitative NMR spectroscopy characterization of EcClpP (29) support this proposal. Nevertheless, the second proposal requires further structural evidence. While this paper was in preparation, a structural study of SaClpP found that the handle helix bent in the tetradecameric packing form, which was suggested to generate pores through that degradative products might escape the proteolytic chamber (30).

Here, we have determined two crystal structures of wild-type SaClpP proteins in two distinct conformations. Although all ClpP structures solved to date are either in the extended or compacted states, these SaClpP structures presented here are the first examples to provide different conformational assemblies for a given ClpP. One structure is very similar to those ClpP structures solved in the extended states; the other is quite different from either the extended or compacted ClpP structures, in that the handle domains adopt compressed conformations. In conjunction with biochemical analyses and molecular dynamic (MD) simulations, these structures provide two different conformational snapshots during ClpP protease dynamic degradation cycle, explain the organizational principles behind biological complex formation, and reveal mechanistic insight into the biological function of the degradation of peptides in the active site, as well as the release of products from the catalytic chamber.

**EXPERIMENTAL PROCEDURES**

**SaClpP Proteins Expression and Purification** - Three forms of SaClpP proteins (N-2-SaClpP, C-His-SaClpP, and mature SaClpP) were constructed for both crystallization and activity studies. N-2-SaClpP protein, which includes 2 more amino acids at the N-terminus, was produced for the crystallization experiment by cloning *S. aureus* Newman strain clpP gene into the *BamH*I and *Xho*I sites of pGEX-4T-1 vector (Novagen) for overexpression in *E. coli* BL21 (DE3) Star cells (Invitrogen). Cells were grown at 37°C, with shaking at 220 rpm, in LB media supplemented with 50 μg/mL Ampicillin to an OD600 of 0.6, at which time protein expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 16°C overnight. Cells were harvested by centrifugation and stored at -80°C until use. A cell pellet was suspended in lysis buffer of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, and 2 mM DTT, and lysed at 4°C by sonication. After centrifugation at 16,000 g for 20 min to remove the insoluble material, the supernatant was loaded onto a 5 mL GST High Performance column (GE Healthcare) and the protein was eluted with a single gradient of 10 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 16°C overnight. Cells were harvested by centrifugation and stored at -80°C until use. A cell pellet was suspended in lysis buffer of 50 mM Tris-HCl (pH 8.0), 100 mM KCl, and 2 mM DTT, and lysed at 4°C by sonication. After centrifugation at 16,000 g for 20 min to remove the insoluble material, the supernatant was loaded onto a 5 mL GST High Performance column (GE Healthcare) and the protein was eluted with a single gradient of 10 mM Glutathione (pH 8.0) in lysis buffer. After thrombin digestion off GST-tag at 4°C overnight, 2 extra amino acids of Gly-Ser were introduced in protein sequence at the N-terminus. The protein was further purified by size exclusion chromatography using a Superdex 200 column (GE Healthcare, 120 mL) in a buffer containing 100 mM KCl, 50 mM Tris-HCl (pH 8.0), and 2 mM DTT.

C-His-SaClpP, the SaClpP with C-terminally His-tagged, was constructed by cloning the corresponding gene into the *Nco*I and *Xho*I sites of pET28b vector (Novagen), and purified through a HisTrap High Performance column (GE Healthcare) and the protein was eluted with a linear gradient of 50-400 mM imidazole. Gel filtration purification was followed to give pure protein that has the native sequence at the N-terminus. The C-terminal 6-His tag was intact for functional study.
The mature SaClpP protein was designed and constructed with N-terminally His-tagged in the NheI and XhoI sites of pET28b vector, so that the tag could be autoproteolytically cleaved during expression to produce protein in native sequence. After cell growth and harvest by a procedure similar to the aforementioned, the supernatant was added to 3 volume of buffer containing 50 mM Tris-HCl (pH 8.0), loaded onto an SP Sepharose cation exchange column (GE Healthcare, 20 mL), that had been equilibrated with buffer A, and eluted with a linear gradient of NaCl (0-1.0 M). The combined fractions (10 mL per fraction) were treated with 1.0 M ammonium sulfate, and loaded onto a Phenyl Sepharose High Performance column (GE Healthcare, 5 mL). The flow-through fractions were collected and concentrated to 2 mL, and then subjected to gel filtration purification using a Superdex 200 column.

**Size Exclusion Chromatography**- Gel filtration was performed at 4°C using a calibrated Superdex 200 HR 10/300 column (GE Healthcare) attached to an AKTA fast protein liquid chromatography system (GE Healthcare). The column was equilibrated with either a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM KCl, and 2 mM DTT, or a buffer using salt condition of 50-200 mM sodium sulfate instead of 100 mM KCl. Molecular mass standards (Bio-Rad) used are: Thyroglobulin (670 kDa), γ-Globulin (158 kDa), Ovalbumin (44 kDa), Myoglobin (17 kDa), and Vitamin B₁₂ (1.35 kDa). Protein was monitored by absorbance at the wavelength of 280 nm.

**Enzymatic Activity Assay**- For peptidase activity (27), the model substrate peptide N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (Suc-LY-AMC) was purchased from Shanghai GL Biochem Ltd, and directly used without further purification. Measurements of the hydrolysis rate of the labeled peptide were performed on 2104 Envision Multilabel Reader (Perkin Elmer). Typically, 20 μg SaClpP proteins in 100 μL of buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM KCl, and 2 mM DTT were incubated at 32°C for 5 min and then 0.5 mM substrate peptide Suc-LY-AMC was added in the reaction system. The fluorescence increase was continuously monitored for 100 min, and the excitation wavelength of 340 nm and the emission wavelength of 450 nm were used. Kinetic assays were performed in triplicates, and kinetic constants were determined with the GraphPad Prism 5 Software by plotting the enzyme velocity against substrate concentration.

In order to further investigate and compare the proteolytic activity of these SaClpP proteins, we carried out protein degradation assays by using two model substrates, GFP-SsrA and β-casein, as described previously (31). Briefly, each run contained 0.6 μM SaClpX, 0.33 μM SaClpP, and 5.0 μM GFP-SsrA or 15.0 μM β-casein in a buffer containing 25 mM HEPES (pH 7.6), 100 mM KCl, 20 mM MgCl₂, 1 mM EDTA, 2 mM DTT, and 10% glycerol in a total volume of 100 μL, where control reactions were performed in the absence of SaClpP proteins. All reactions were monitored at 37°C for 40 min or 90 min by separating the proteins on SDS-PAGE that were Coomassie stained.

**Crystallization, Data Collection, and Structure Determination**—Crystallization of purified N-2-SaClpP protein was performed using the hanging drop vapor diffusion method at room temperature. Typically, the crystallization drop was prepared by well mixing a 2 μL protein [10 mg/mL in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl] and equal volume of reservoir solution, and then equilibrated against 500 μL of reservoir solution. Sphenoid shaped crystals grew up in a reservoir solution containing 100 mM citric acid (pH 3.5), and 2.0 M ammonium sulfate within 2 days. Hexagonal rod-like crystals appeared in the reservoir solution of 10% (w/v) PEG 3000, 100 mM cacodylate (pH 6.5), and 200 mM MgCl₂. The crystals were mounted and flash-frozen in liquid N₂ following cryoprotection with the reservoir solution containing an extra 10-20% glycerol. Diffraction data were collected at Shanghai Synchrotron Radiation Facility (SSRF) beamline 17U. All X-ray data were processed using HKL2000 program suite (32) and converted to structure factors within the CCP4 program (33). The crystals belong to the space group P6₃22 and C₂, respectively. The structures were phased by molecular replacement in Phaser (34), using previously published BsClpP truncated monomer as search model (PDB code 3KTG). The N-2-SaClpP model was manually built using COOT (35) and computational refinement was carried out with the program REFMAC5 (36) in
Molecular Dynamic Simulations- MD simulations were performed on two structures: one monomer extracted from SaClpP tetradecamer (SaClpP\textsuperscript{mono}) and one heptameric ring from SaClpP tetradecamer (SaClpP\textsuperscript{14half}). The missing N-terminal residues of some monomers were added by PyMOL. Gromacs software package version 4.5.3 (38) and the AMBER03 force field (39) in explicit TIP3P water (40) were used to run MD simulations. The water box was 10 Å from the protein on all sides (i.e. the starting structure had 20 Å between periodic images). Hydrogen bonds were constrained using the linear constraint solver (LINCUS) algorithm (41). Na\textsuperscript{+} ions and Cl\textsuperscript{−} ions were added to neutralize the simulation systems. Long range electrostatic interactions were treated by using the particle-mesh Ewald method (42). Periodic boundary conditions were applied to avoid edge effects in all calculations. The temperature was kept constant at 300 K by using the Berendsen thermostat method (43) with a coupling time of 1 ps. Berendsen pressure coupling (43) was used for equilibration of the systems.

To investigate the conformational transitions of SaClpP protease implied by these two structures, we carried out 200 ns MD simulations on SaClpP\textsuperscript{mono} and SaClpP\textsuperscript{14half}, respectively. Before the MD simulation run, the systems were subjected to energy minimizations using the steepest-descents algorithm (44). Then, the systems were heated gradually from 0 K to 300 K. First, 100 ps simulation was carried out to heat the solvent molecules and ions. Following that, 50 ps and 20 ps simulations were performed to heat all the atoms in the system with the restriction of main chain and protein Ca atoms, respectively. Finally, the conventional MD was performed with coordinates saved every 10 ps during the entire process.

RESULTS

Oligomeric State and Degradative Activity of SaClpP in Solution- In the size exclusion chromatography, the retention time of N-2-SaClpP protein under 100 mM KCl is between those of tetradecameric and heptameric EcClpP (supplemental Fig. 1A), suggesting that the oligomeric state of N-2-SaClpP is in equilibrium in heptameric and tetradecameric forms. However, both the mature SaClpP and C-His-SaClpP proteins display tetradecameric organization under the same buffer conditions (Fig. 1A). The EcClpP double rings could be reversibly dissociated in the presence of sodium sulfate (45). To test whether this would also happen on SaClpP, we conducted a gel filtration assay for all three proteins in a buffer containing 200 mM sodium sulfate. Consistent with previous observation in EcClpP, the stability of the double heptameric rings of wild-type SaClpP is also dependent on salt conditions (Fig. 1A). Moreover, treatment of the native C-His-SaClpP protein with sodium sulfate causes the tetradecameric oligomerization to migrate towards a heptameric state in a salt concentration-dependent manner (supplemental Fig. 1B).

As shown in Fig. 1B, two SaClpP proteins having native amino acid sequences at the N-terminus (mature SaClpP and C-His-SaClpP) and the protein with two extra amino acids at the N-terminus (N-2-SaClpP) are all active for efficient degradation of the short model peptide Suc-LY-AMC in a comparable range of activity in vitro. The measured $K_m$ and $K_{cat}$ values are 145 μM and 225 min\(^{-1}\) for mature SaClpP, 133 μM and 146 min\(^{-1}\) for C-His-SaClpP, and 151 μM and 151 min\(^{-1}\) for N-2-SaClpP, respectively (Fig. 1B). Interestingly, this peptidase activity appears dependent on sulfate concentration. The $K_m$ stays almost constant (between 133 and 158 μM), but $K_{cat}$ increases ~3 folds (from 145 to 456 min\(^{-1}\)) as the sodium sulfate concentration is raised to 200 mM (supplemental Fig. 1C and supplemental Table 1), indicating that the substrate peptides binding by SaClpP is not affected by the sulfate ion. However, the enzyme catalytic turnover is increased in the presence of sulfate.

Consistent with previous reports on EcClpP (15), the N-terminal 6-His tag is able to undergo autoproteolysis by SaClpP protease itself during protein expression (supplemental Fig. 1D). In the degradation of the model substrate proteins GFP-SsrA and β-casein in the presence of SaClpX, the N-2-SaClpP protein shows lower proteolysis activity compared to the native protein.
C-His-SaClpP under the same reaction conditions (Fig. 1C and supplemental Fig. 1E). This result is possibly due to its organizational equilibrium between tetradecameric and heptamer. This observation suggests that the tetradecameric organization of ClpP assembly is necessary for degradation of substrate protein.

**Overall Structure of the Extended SaClpP**

The extended SaClpP was crystallized in space group C2. The structure was refined at 2.28 Å resolution (Table 1). In this crystal, the asymmetric unit consists of two heptameric rings stacking face-to-face to form a tetradecamer. Because ClpPs display a high degree of sequence similarity in various organisms (Fig. 2), it is no surprise that the overall protein folding of SaClpP closely resembles the common feature of the previously solved ClpP structures in the extended states from other organisms (Fig. 3A and supplemental Fig. 2, A and B) (9-19,21).

Structurally, a SaClpP monomer can be divided into three parts: N-terminal loop (colored in green), head domain (yellow), and handle domain (cyan) (Fig. 3A). Functionally, the N-terminal loops bearing seven antiparallel β-hairpins (Fig. 3D) control the entrance diameter of the protease chamber. In this extended SaClpP, the loops fill the space surrounding the entrance to the axial channel in defining a narrow pore about 12 Å in diameter (Fig. 5A, bottom panel), to allow only the passage of a single amino acid (16). The head domain comprises the bulk of the protein and forms the apical surface of the tetradecamer. The handle domains in each heptamer consist of one β-strand and a long helix (αE), which intercalates with the handle domains of the opposing heptameric ring to enclose a tetradecameric cylinder of SaClpP.

**Arg171_Asp170 Network Keeps Ring-Ring Interlocking and Handle Helix Extended**

It was previously proposed that the ring-ring stacking in ClpP tetradecamer was mainly stabilized by charge-charge interaction from the head domain (23,45). In EcClpP, truncations in the handle domain did not lead to dissociation of the double rings. However, the mutation of Arg 184 or Glu 183, corresponding to Arg 171 or Asp 170 in SaClpP, respectively, caused ring-ring dissociation (23). Similarly, in the extended SaClpP tetradecamer, the two heptameric rings are held together by extensive hydrogen bonding and salt bridge interactions mediated through an Arg171_Asp170 network from apposing subunits (Fig. 3B). It is worth noting that these interactions are just located at equator of the tetradecameric cylinder (Fig. 3A), so electrostatic interactions not only exist between the two oppositely charged residues from the same monomer, but also between those from apposing monomers. Besides electrostatic interactions, the guanidino side chain of Arg171 is located close to several polar residues, such as Gln 124, Gln 132, and Glu 135 from other monomers, which are participating in an interdependent bonding network (Fig. 3B). Furthermore, residues Gln 132 and Glu 135, located at top tip position of helix E, form hydrogen bonds with Arg 171 and Asp 170 from a neighboring subunit, which is most likely to play a significant role in keeping helix E in a straight conformation. The highly conserved orientations of Arg 171 and Asp 170 in all seven monomers from one ring further demonstrate the functional importance of these two key residues (Fig. 3C). Taken together, the Arg171_Asp170 network seems to be crucial not only to maintain the cylindrical chamber enclosed, but also to predominantly keep the handle helix in a straight conformation.

**Overall Structure of the Compressed SaClpP**

The crystal structure of the compressed SaClpP was solved and refined at 2.43 Å resolution (Table 1). In this crystal, each asymmetric unit consists of one single heptameric ring, as half of that shown in Fig. 4A. The overall protein folding is strikingly different from either the extended or compacted states as observed in ClpPs from other species (supplemental Fig. 2, C and D). The N-terminal loops are completely unstructured, opening a wide axial pore ~20 Å in diameter at the N-terminus (Fig. 5B, bottom panel). In particular, the handle helix displays substantial differences from the extended SaClpP (Fig. 3A), implying very important functional differences between these two states. To gain the interactions around the ring-ring interfaces in the compressed structure, the kind of tetradecameric packing observed in the crystal is built by symmetric operation according to packing lattice. In contrast to the tightly packed tetradecameric structure of the extended state (Fig. 3A), all salt bridge and hydrogen-bonding interactions along the equator of the catalytic chamber disappear in the
compressed tetradecamer of *Sa*ClpP (Fig. 4B). It is also found that the side chains of Asp 170 and Arg 171, which were involved in holding two rings stacked closely in the extended *Sa*ClpP, are positioned in disordered orientations (Fig. 4C and supplemental Fig. 3). The ring-ring interfaces are therefore significantly weakened in the compressed structure. Moreover, some residues are observed in electrostatic repulsion positions along the equator (Fig. 5B, top panel and supplemental Fig. 4, A and C). All these structural elements may result in loose contacts between the double heptameric rings in the compressed *Sa*ClpP assembly.

**Handle Helix Kinks to Push Ring Compressed-** In the extended *Sa*ClpP, salt bridge contacts between Asp 170 and Arg 171 from both rings play crucial roles to lock ring-ring stacking into a tetradecameric organization, and keep the handle helix E anchored in an extended conformation (Fig. 3, A and B). However, we found that entire handle motifs were broken at residues Lys 145, showing bent conformations in the compressed *Sa*ClpP structure (Figs. 4D and 6A). One consequence of this reengagement is a large loss of ring-ring buried binding surface. The buried surface area between the double rings in the extended structure is calculated to be around 12800 Å² in PyMOL, 25% larger than that in the compressed *Sa*ClpP (10000 Å²). The imperfect packing of two heptameric rings in the compressed state indicates potent dissociation of double heptameric rings for product release. Therefore, the interesting question to address is how the handle motifs maintain kinked conformations in the compressed *Sa*ClpP. Through examining the structure, we discovered that a hydrogen-bonding network exists in order to keep the helix E in a kinked conformation (Fig. 4D). The polar side chain of Glu 137, located at the top tip position of the helix E, participates in the formation of extensive hydrogen bonding with neighboring residues including Asp 38, Ser 70, and Thr 72, which are all located in the stable conformational head domain.

The overall dimensions of the extended *Sa*ClpP tetradecamer are 96 Å in height (for head and handle domains) and 100 Å in diameter (Fig. 5A). The two compressed heptameric rings are packed in overall dimensions of 84 Å in height and 108 Å in diameter (Fig. 5B). In order to understand the structural element in the formation of the compressed *Sa*ClpP cylinder, we performed three superimpositions in PyMOL (Fig. 5, C-E). Two single monomers, extracted from heptamer and tetradecamer, respectively, were overlaid to give a root mean square deviation (RMSD) of 0.6 Å. This small value reflects a close overall similarity of monomeric architectures; in particular, the head domains are almost identical in these two structures (Fig. 5C). However, the assembly of seven kinked monomers into one heptameric ring severely compresses the overall dimension by about 6 Å in height. To further investigate how this happens, we performed two more independent ring-ring overlays. Fig. 5D shows the result of that a heptameric ring is overlaid with one ring from *Sa*ClpP tetradecamer, while Fig. 5E shows the result of overlaying the two rings by using two specific monomers as a reference. It can be clearly seen that the long helix E is broken into two short helices at the position of residue Lys 145, and the newly formed short helix is laying down in order to push away the neighboring monomer, avoiding rigid body constraints (Fig. 5, D and E). Consequently, the overall packing of the compressed tetradecamer becomes wider in diameter compared to that of the extended *Sa*ClpP tetradecamer. Through taking this compressed conformation, *Sa*ClpP squeezes the chamber space for substrate binding, showing it is in an inactive conformation for catalytic degradation.

**Conformational Changes Observed in Molecular Dynamic Simulation-** As shown in Fig. 6A, the two positions of helix E represent two distinct conformations of *Sa*ClpP. Within the ~80 degrees fluctuating angle in width, many snapshot configurations possibly exist accompanied by the catalytic degrading function. To investigate the dynamic features, we monitored root mean square fluctuation (RMSF) along MD trajectories. RMSF reflects the mobility of a certain residue around its average position. The RMSF values of each residue in ClpPmono indicated that large fluctuations of residues occurred in sequence between His 123 and Lys 145, which is the kinked part of the handle domain in the compressed *Sa*ClpP (Fig. 6B), suggesting that this part is very flexible and that the kink is spontaneous. To assess the dynamic stability of the two systems during the simulations, we also monitored RMSD values of
protein Cα atoms relative to the initial X-ray crystal structure along the entire MD trajectories (Fig. 6C). The structure of ClpP14half appears to be more stable than ClpPmono, indicating that monomers in heptameric ring can stabilize each other.

As discussed above, Arg 171 and Asp 170 from opposing rings can interact with and stabilize each other in the extended ClpP structure, keeping helix E straight. Since there is only one single ring in ClpP14half, Arg 171 and Asp 170 cannot be stabilized by those from opposing rings, so we can assume that the Arg171_Asp170 interaction network, which exists in the extended state, disappears in ClpP14half. Thus, the MD simulation results reveal dynamic properties of ClpP without this network. RMSF profiles of ClpP14half indicate that the residues with higher fluctuation values are His 123 - Lys 145 in the handle domain and helix F. The residue with the largest RMSF value is Ala 133, which is located at the top tip position of helix E (Fig. 6B, red box). Accordingly, superimposition of the compressed state with the extended state reveals kinking of helix E and a major shift in helix F (Fig. 5C and supplemental Fig. 2D). Furthermore, we examined the snapshots isolated from the trajectory of ClpP14half (Fig. 6D). The MD result indicates that His 123 shifted away from the active conformation and gradually flipped to another orientation. Taken together, the MD results suggest that the kink in helix E is mechanically spontaneous. Without the Arg171_Asp170 network, the extended SaClpP shows a remarkable tendency toward the compressed state.

ATPase-Binding Pockets Closed in the Compressed SaClpP- A new class of antibiotics, ADEPs, have been identified to activate ClpP in the absence of ATPases (15). Recently, two independent structural studies have provided the molecular mechanisms of ADEPs binding in hydrophobic pockets of BsClpP and EcClpP (18,20). As shown in Fig. 7A, in the activated EcClpP (PDB code 3MT6), the long aliphatic side chain of ADEP1 mimics the loop of ATPases to properly fit into the hydrophobic channel on surface (18). To gain the conformational differences in ATPase-binding pockets, we overlaid the two SaClpP structures on the ADEP1-activated EcClpP structure. As shown in Fig. 7B, the aliphatic-chain binding channel is almost fully opened in the extended SaClpP. We observed the similar result in the EcClpP-ADEP1 complex structure. The corresponding binding pocket, however, is completely closed to make ADEP1 side-chain binding impossible in the compressed SaClpP (Fig. 7C). In the overlaying model, the aliphatic side chain of ADEP1 is entirely buried inside the protein surface. These models suggest that dynamic motion of the protease catalytic core also induces the conformational change of the hydrophobic pocket for ATPase-binding.

Catalytic Triads Observed in the Extended and Compressed SaClpP- In the structure of HpClpP-peptide complex (PDB code 2ZL2) (19), along the axial direction, the alignment of key functional regions include three parts: the upper catalytic triad includes Ser 99, His 124, and Asp 173 in one monomer, corresponding to Ser 98, His 123, and Asp 172 in SaClpP respectively; the interlocking region includes Asp 171, Arg 172, and Asp 136, corresponding to Asp 170, Arg 171, and Glu 135 in SaClpP respectively, connecting four neighboring monomers; and the lower catalytic triad has same residues as in the upper from the opposing monomer (Fig. 8A). These three regions are physically located close to each other. For the extended SaClpP, the orientations of the catalytic triads are quite similar to those of the structure of HpClpP-peptide complex (Fig. 8B). Each SaClpP catalytic triad (9), containing residues Ser 98, His 123, and Asp 172, is located at the junction parts of the head and handle domains, and quite close to the ring-ring interlocking regions (Fig. 8B). Therefore, the interaction between Arg 171 and Asp 170 would impinge on the catalytic triad when conformational change occurs. In the compressed SaClpP, the underlying secondary structural elements necessary for the proper orientations of the residues in the catalytic triad are completely dispositional (Fig. 8C): Asp 172 shifts away from the rest of the triad, and His 123, as a consequence of the repacking, is flipped into an orientation where it can no longer bridge Ser 98 and Asp 172 by hydrogen bonding. This reengagement of the active site residues causes the loss of substrate-binding positions, suggesting that the compressed SaClpP is in a proteolytically inactive state.
Proposed Conformational Switching between the Extended and Compressed States—Spacious pockets for substrate peptides binding exist inside the chamber of the extended SaClpP (Fig. 8B). The catalytic triad residues can properly bind to substrates through hydrogen-bonding with Ser 98 and His 123. After catalytic cleavage of the peptide bond, the accumulation of digested segments in the active site may induce the rearrangement of the hydrogen-bonding network nearby. As a result, His 123 shifts away from the active position and is gradually flipped to another orientation, leading to the destruction of the Arg171_Asp170 contact network (Fig. 8C). The restraint for helix E by interactions between Glu 135, Gln 132, and Arg 171 are released, enabling it to undergo a spontaneous conformational switch; the peptide-binding pocket disappears with it because the kinked helix E is no longer properly packing as a part of the substrate-binding pocket. More intriguing is the possibility that ionic changes in the active cavity upon peptide bond cleavage might affect the interacting contacts, leading to partial dissociation of the two rings or other conformational changes that would release products outside the chamber.

DISCUSSION

The crystal structures of many members of the ClpP family have been described (9,16-25,30). Of those, only EcClpP structures have been solved both in the extended and compacted states for wild-type and disulphide cross-linked proteins, respectively. Such structures indicate that the handle domain may naturally undergo a conformational change during the course of dynamic transitions between the extended and compacted states (22). However, how the degraded peptides products exit the catalytic chamber still remains unclear since there is no direct structural evidences to exclusively support the hypothesis of product release through the handle domain. In this study, we report the structural characterizations of SaClpP in two distinct conformations. It is the first time that two distinct states have been observed in wild-type ClpP protease for a specific organism. This discovery enabled us to investigate the molecular mechanisms of substrates degradation and product release.

The observation (supplemental Fig. 1C and supplemental Table 1) that the native SaClpP degrades the model peptide Suc-LY-AMC in 200 mM sodium sulfate at triple the rate of that exhibited by the same protein in buffer containing 100 mM KCl suggests that heptameric organization is much efficient for short peptide digestion in vitro. Similar to EcClpP (45), SaClpP appears a typical heptameric oligomerization in the presence of 200 mM sodium sulfate (Fig. 1A and supplemental Fig. 1B). This organization probably allows substrate peptides easier access into the active sites through C-terminal opening than through N-terminal pores. Most interestingly, the heptameric oligomerization of SaClpP appears to correlate well with its peptidase activity (supplemental Fig. 1, B and C), further suggesting that peptide degradation is much efficient in the opened chamber of ClpP. On the other hand, a tetradecameric assembly of ClpP is necessary for protein degradation in ATPase-dependent manner. This is supported by the observation (Fig. 1C and supplemental Fig. 1E) that N-2-SaClpP, which has 2 extra amino acids at N-terminus, has lower proteolysis activity on model protein degradation compared to that of C-His-SaClpP which has native amino acid sequence at the N-terminus. Given the oligomeric equilibrium between tetradecameric and heptamer of N-2-SaClpP protein (Fig. 1A and supplemental Fig. 1A), the relative lower proteolytic activity seems to support the notion that tetradecameric organization is required for protein degradation in the presence of ATPase, such as ClpX or ClpA. The molecular mechanisms behind these observations remain enigmatic and await further investigations.

The N-terminal sequences of over 100 ClpP proteins derived from different prokaryotic and eukaryotic genomes, show a very high degree of conservation (Fig. 2) (7). If well-packed or disordered N-terminal loops modulate peptide diffusion into the ClpP chamber by tuning the diameters of axial pores remains controversial (16,23,46-48). In the ADEP1-bound EcClpP structure (PDB code 3MT6), all the N-terminal loops adopt well-ordered β-hairpin conformations that maximally open an axial pore about 20 Å in diameter (18), allowing unfolded proteins to enter into active sites for proteolysis. In the extended SaClpP, the overall protein folding reserves the common features of ClpP structures in the
extended organization (9,16-21). Some N-terminal β-hairpins are well-ordered in the extended SaClpP (Fig. 3D), showing how these loops participate in controlling the narrowness of the pores and restrict the entrance of large polypeptides into the axial channel. The other loops are partially disordered like those observed in previously solved structures, indicating that these regions are usually conformationally flexible and dynamic. The crystal packing pattern and molecule contact in crystal might contribute to the partial-ordered conformation of those in the observed N-terminal loops. All the N-terminal loops in the compressed SaClpP structure are completely unstructured (Fig. 4A), indicating that ATPase-binding nearby N-terminus might enable these loops to assume a well-ordered conformation. The breathing motions of N-terminal regions, accompanied by other conformational changes, are likely to account mainly for controlling the entry of substrate proteins into the catalytic chamber of ClpP.

It has been established that charge-charge interaction plays an important role for ring-ring stacking in the formation of tetradecameric cylinder (23). Indeed, close inspection of the extended SaClpP shows that polar residue Arg 171 plays a central role through the formation of an extensive network of salt bridge and hydrogen bonding with surrounding residues. These bridges and bonds link the four neighboring monomers such that they will closely interact with each other (Figs. 3B and 8B). Further, Glu 135 and Asn 132, located on the top tip position of the turn in the handle domain, prevent long helix E from kinking away by hydrogen bonding to Arg 171 and Asp 170 (Figs. 3B and 6A). These interactions are critical to keeping the long handle helix straight. Based on our structural analysis, it is tempting to suppose that disruption of these elaborate interactions contributes to the dynamic conformational changes between the extended and compressed states that accomplish the degradation cycle in the cylindrical protease.

The protein crystallized in ammonium sulfate as a tetradecamer with single heptamer in asymmetric unit. However, the compressed structure shows the tendency of ring-ring dissociation under the following reasons. First, the high concentration in crystal could have promoted the formation of double rings. In addition, the N-2-SaClpP protein exists in a dynamic equilibrium between heptameric and tetradecameric forms, confirmed by gel filtration (Fig. 1A). Treatment of native SaClpP protein with sodium sulfate allows for the tetradecamer migrating towards heptameric form in a concentration-dependent manner (supplemental Fig. 1B). A similar phenomenon has been reported for EcClpP (45). Furthermore, it has been established that mutation of Arg184_Glu183 interaction network leads to dissociation of the double heptameric rings in EcClpP (23), and the corresponding Arg171_Asp170 network appears weakened in crystal structure of compressed SaClpP (Fig. 4B and supplemental Fig. 3). Moreover, in the built compressed tetradecamer, the buried binding surface between the double rings was significantly reduced compared to that in the extended SaClpP tetradecamer, and some residues were observed in electrostatic repulsion positions (Fig. 5A and supplemental Fig. 4, A and C). Similar to the compressed SaClpP, the structures of PfClpP and MtClpP have been solved as compacted tetradecamers in crystals. However, these proteins can exist as stable heptamers under physiological conditions, which were confirmed by analytical ultracentrifugation and size-exclusion chromatography (24,25).

In the compressed SaClpP, the conformation of handle domain is very different from that of the extended SaClpP tetradecamer (Figs. 3 and 4). Typically, the long helix E is packed in a disordered manner in all ClpP structures solved in the compacted states (22-25). Unexpectedly, in the compressed SaClpP structure, the long helix motif adopts a stable configuration, broken severely into two helices at the residue Lys 145 (Fig. 6A). Judged as normal through model analyses, this significant conformational change seems to be initiated by disruption of the extensive Arg171_Asp170 interactions network, which is located around the interfaces of double rings, and quite close to catalytic triads. The disappearance of the interactions between Glu 135 (and Gln 132) and Arg 171 causes the handle helix to lose the force of its anchor at the extended state, and to swing spontaneously away from the active site. This predominant motion is likely to result in partial dissociation of double ClpP rings in the stacked tetradecamer, therefore allowing short peptides or free amino acids to escape the catalytic
chamber. This notion is further supported by MD simulation experiments (Fig. 6, B and C). The MD result establishes that handle region is indeed dynamic, and kink of helix E is mechanically spontaneous. Without Arg171 Asp170 network restraint, the extended SaClpP shows a remarkable tendency to switch to the compressed status. In addition to the MD experiment, we also applied a biochemical assay to test the stability of the ring-ring stacking in solution. The concentration of sulfate ion drives the movement of self-assembly between tetradecamer and heptamer (Fig. 1A and supplemental Fig. 1B), revealing that ionic strength may influence the stability of double heptameric rings’ packing. Initially, such movement might be induced by the dynamic fluctuation of the handle motif from an extended orientation.

ClpP protease by itself has limited degradative activity on small peptides (26,27,49) and some poorly folded proteins (47,48). The formations of ClpAP and ClpXP complex are key for the degradation of large protein substrates (50). To achieve complex formation, ATPases utilize the highly conserved tripeptide consensus sequence of IGF/L to specifically dock into the hydrophobic pockets of the N-terminus of ClpP (51). The dynamic structural switching is also associated with conformational changes of these hydrophobic pockets. Compared to models of EcClpP or BsClpP bound with AEDP1 (18,20), the extended SaClpP structure shows a similar pocket feature, which could be viewed as a state in which the binding pocket is almost fully opened and ready to specifically accommodate the binding of flexible loops of ClpX (Fig. 7, A and B). However, the shape of pocket changes significantly in the compressed SaClpP (Fig. 7C). There is not enough open space for binding by aliphatic loops of ClpX, indicating that the compressed SaClpP is in an inactivated status for catalytic degradation. This observation is similar to the previous result that the compacted EcClpP did not bind ClpX (22). Judging not only based on the dynamic conformational change in the handle domains, but also from the structural differences in ATPase-binding pockets, the dynamic switching between these two structures describe the course of the functional cycle of this proteolysis system.

The compressed SaClpP structure has suggested that ring-ring dissociation is also likely to occur in the entire catalytic cycle of ClpP protease. The long helix E is found to be in either straight or kinked conformation in the extended or compressed SaClpP structure, respectively. The dynamic window for the conformational fluctuation is broad up to ~80 degrees between these two states (Fig. 6A). However, it would not be necessary to shift helix E to the fully bent position, and leave the chamber completely open to solvent. MD analysis of both monomeric and heptameric SaClpP structures indicates that any snapshots in the continuous conformational change possibly occur spontaneously, accompanied by other structural changes to accomplish the entire cycle from substrate entry to product release. In the compressed SaClpP tetradecamer, the two heptameric rings are rotated with respect to each other by eight degrees around the axial directions, while the corresponding rotation angle was estimated to be five degrees in the compacted EcClpP tetradecamer (23), indicating that the compacted structure seems to represent an intermediate status of transition between the extended and compressed states (supplemental Fig. 2, C and D). Overall, the extended conformation of helix E is critical to facilitate the two heptameric rings to stack face-to-face in the formation of a tetradecamer for substrate degradation. Meanwhile, the transient fluctuation of the handle domain is a key to understand the mechanism of products release.

Mechanistically, in conjunction with these structural characterizations, further MD simulation experiments draw connection between the conformational change in active site and helix E kinking during the entire structural switching. These experiments reveal the whole process of substrates degradation in catalytic triads and products release from catalytic chamber (Fig. 8). In the extended active state, His 123 and Asp172 involved in catalytic triad formation align themselves in proper geometry by bridging residue Ser 98. However, the side chains of these residues flip away from the active site and are positioned toward the charge-charge interlocking region after substrate proteolysis and then ClpP starts to switch to the compressed state. Consequently, the participation of these polar residues destroys the central interaction networks of Arg171 Asp170. Therefore, the long helix kinks away as a result of loss of hydrogen-bonding restraint on the top tip,
thus leading to partial dissociation of the two heptameric rings or other conformational changes that would release products outside the chamber, depending on how much the kinking of helix E undergoes.

In summary, these two structures of \textit{Sa}ClpP, together with biochemical assay and MD simulation data, have provided two different conformational snapshots of ClpP undergoing structural switch in order to perform a catalytic functional cycle. Furthermore, the structures explain the organizational principles behind biological complex formation and reveal mechanistic insight into the biological function of peptides degradation in the active site, as well as products release from the cylindrical chamber. The fundamental understanding of the structural features in two different functional states presented here can now be used to further investigate the molecular mechanism of substrate translocation into the hollow chamber of ClpP in the presence of ATPase.

REFERENCES

1. Gottesman, S., Maurizi, M. R., and Wickner, S. (1997) \textit{Cell} 91, 435-438
2. Gottesman, S., Wickner, S., and Maurizi, M. R. (1997) \textit{Genes Dev} 11, 815-823
3. Wawrzynow, A., Wojtkowiak, D., Marszalek, J., Banecki, B., Jonsen, M., Graves, B., Georgopoulos, C., and Zylicz, M. (1995) \textit{Embo J} 14, 1867-1877
4. Porankiewicz, J., Wang, J., and Clarke, A. K. (1999) \textit{Mol Microbiol} 32, 449-458
5. Chandu, D., and Nandi, D. (2004) \textit{Res Microbiol} 155, 710-719
6. Butler, S. M., Festa, R. A., Pearce, M. J., and Darwin, K. H. (2006) \textit{Mol Microbiol} 60, 553-562
7. Yu, A. Y., and Houry, W. A. (2007) \textit{FEBS Lett} 581, 3749-3757
8. Corydon, T. J., Bross, P., Holst, H. U., Neve, S., Kristiansen, K., Gregersen, N., and Bolund, L. (1998) \textit{Biochem J} 331 (Pt 1), 309-316
9. Wang, J., Hartling, J. A., and Flanagan, J. M. (1997) \textit{Cell} 91, 447-456
10. Chatterjee, I., Becker, P., Grundmeier, M., Bischoff, M., Somerville, G. A., Peters, G., Sinha, B., Harraghy, N., Proctor, R. A., and Herrmann, M. (2005) \textit{J Bacteriol} 187, 4488-4496
11. Frees, D., Chastanet, A., Qazi, S., Sorensen, K., Hill, P., Msadek, T., and Ingmer, H. (2004) \textit{Mol Microbiol} 54, 1445-1462
12. Frees, D., Qazi, S. N., Hill, P. J., and Ingmer, H. (2003) \textit{Mol Microbiol} 48, 1565-1578
13. Michel, A., Agerer, F., Hauck, C. R., Herrmann, M., Ullrich, J., Hacker, J., and Ohlsen, K. (2006) \textit{J Bacteriol} 188, 5783-5796
14. Donegan, N. P., Thompson, E. T., Fu, Z., and Cheung, A. L. (2010) \textit{J Bacteriol} 192, 1416-1422
15. Brotz-Oesterhelt, H., Beyer, D., Kroll, H. P., Endermann, R., Ladel, C., Schroeder, W., Hinzen, B., Raddatz, S., Paulsen, H., Henninger, K., Bandow, J. E., Sahl, H. G., and Labischinski, H. (2005) \textit{Nat Med} 11, 1082-1087
16. Bewley, M. C., Graziano, V., Griffin, K., and Flanagan, J. M. (2006) \textit{J Struct Biol} 153, 113-128
17. Szyk, A., and Maurizi, M. R. (2006) \textit{J Struct Biol} 156, 165-174
18. Li, D. H., Chung, Y. S., Gloyd, M., Joseph, E., Ghirlando, R., Wright, G. D., Cheng, Y. Q., Maurizi, M. R., Guarne, A., and Ortega, J. (2010) \textit{Chem Biol} 17, 959-969
19. Kim, D. Y., and Kim, K. K. (2008) \textit{J Mol Biol} 379, 760-771
20. Lee, B. G., Park, E. Y., Lee, K. E., Jeon, H., Sung, K. H., Paulsen, H., Rubsam-Schaeff, H., Brotz-Oesterhelt, H., and Song, H. K. (2010) \textit{Nat Struct Mol Biol} 17, 471-478
21. Kang, S. G., Maurizi, M. R., Thompson, M., Mueser, T., and Alvazie, B. (2004) \textit{J Struct Biol} 148, 338-352
22. Kimber, M. S., Yu, A. Y., Borg, M., Leung, E., Chan, H. S., and Houry, W. A. (2010) \textit{Structure} 18, 798-808
23. Gribun, A., Kimber, M. S., Ching, R., Sprangers, R., Fiebig, K. M., and Houry, W. A. (2005) \textit{J Biol Chem} 280, 16185-16196
24. El Bakkouri, M., Pow, A., Mulichak, A., Cheung, K. L., Artz, J. D., Amani, M., Fell, S., de Koning-Ward, T. F., Goodman, C. D., McFadden, G. I., Ortega, J., Hui, R., and Houry, W. A. (2010) *J Mol Biol* **404**, 456-477
25. Ingvarsson, H., Mate, M. J., Hogbom, M., Portnoi, D., Benaroudj, N., Alzari, P. M., Ortiz-Lombardia, M., and Unge, T. (2007) *Acta Crystallogr D Biol Crystallogr* **63**, 249-259
26. Thompson, M. W., and Maurizi, M. R. (1994) *Acta Crystallogr D Biol Crystallogr* **60**, 2126-2132
27. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) *Acta Crystallogr D Biol Crystallogr* **53**, 240-255
28. DeLano, W. L. (DeLano Scientific, Palo Alto, CA, 2002)
29. Wallis, L. D., Bohon, J., Chance, M. R., and Licht, S. (2008) *Biochemistry* **47**, 11031-11040
30. Bewley, M. C., Graziano, V., Griffin, K., and Flanagan, J. M. (2009) *J Struct Biol* **165**, 118-125
31. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) *Bioinformatics (Oxford, England)* **23**, 2947-2948
32. Gouet, P., Courcelle, E., Stuart, D. I., and Metoz, F. (1999) *Bioinformatics (Oxford, England)* **15**, 305-308
33. Rocchia, W. A., E. Honig, B. (2001) *J Phys Chem B* **105**, 6507-6514
Acknowledgments- We thank Dr. Jianhua Gan for crystallographic discussion and assistance, Professor Haiyan Zhang for usage of instruments, Ms. Sarah Frank Reichard for critical reading of the manuscript, and we acknowledge user support at beam line BL17U at Shanghai Synchrotron Radiation Facility and Tianjin Super-computing Center, China.

FOOTNOTES

*Data collection was conducted at BL17U at Shanghai Synchrotron Radiation Facility, China. Molecular Dynamic Simulation was performed at Tianjin Super-computing Center. This work was also supported by National Natural Science Foundation of China (20972173, 20972174, 90913010, 91029704, 21021063, and 21172234), the Hundred Talent Program of the Chinese Academy of Sciences (to C.Y. and L.L.), the Science and Technology Commission of Shanghai Municipality (09PJ1411600 and 10410703900), the Key Project of Chinese National Programs for Fundamental Research and Development (2009CB918502), the National Science and Technology Major Project “Key New Drug Creation and Manufacturing Program” (2009ZX09301-001), and Special Grant of the Chinese Academy of Sciences (XDA01040305).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1-4 and Table 1.

The atomic coordinates and structure factors (codes 3ST9 and 3STA) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1These authors contributed equally to this work.

2To whom correspondence may be addressed: Tel.: 86-21-50806600; Fax: 86-21-50807088; E-mail: cluo@mail.shcnc.ac.cn.

3To whom correspondence may be addressed: Tel.: 86-21-50806029; Fax: 86-21-50807088; E-mail: yangcg@mail.shcnc.ac.cn.

The abbreviations used are: Sa, Staphylococcus aureus; Ec, Escherichia coli; Bs, Bacillus subtilis; Hp, Helicobacter pylori; Pf, Plasmodium falciparum; MD, molecular dynamic; Suc-LY-AMC, N-succinyl-Leu-Tyr-7-amido-4-methylcoumarin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; LINCS, linear constraint solver; RMSD, root mean square deviation; RMSF, root mean square fluctuation.

FIGURE LEGENDS

FIGURE 1. Organizational property and enzymatic activity of SaClpP variants. A. Size exclusion chromatographic analysis of oligomeric organization of three SaClpP proteins. Protein peaks are monitored by UV absorbance at wavelength of 280 nm and retention volumes corresponding to molecular mass are recorded in 100 mM KCl and 200 mM sodium sulfate, respectively. The molecular mass standards are indicated on top of the figure. B. Hydrolysis of the fluorogenic peptide Suc-LY-AMC by three SaClpP proteases. Kinetic constants are determined with the GraphPad Prism 5 Software by plotting enzyme velocity against substrate concentration. Error bars are shown based on three independent repeats. C. GFP-SsrA proteolysis by C-His-SaClpP or N-2-SaClpP under the same conditions is shown on SDS-PAGE gel stained with Coomassie Blue. Each reaction is monitored for 40 min, and control runs without ClpP are shown in the last two lanes.
**FIGURE 2.** Sequence alignment and secondary structure assignment of ClpP proteases. Sequence alignment is performed in ClustalW2 (52) and drawn with ESPript (53). The identical residues are highlighted in red, while those residues in red font are highly homologous. Residues in catalytic triad are marked below by an asterisk. Secondary structure elements present in the extended SaClpP structure (PDB code 3STA) are shown on top of the sequence alignment, with residue numbers at top of the alignment following SaClpP.

**FIGURE 3.** Crystal structure of the extended SaClpP. A. Cartoon of the overall SaClpP tetradecamer. The N-terminal loops are colored in green, head domain in yellow, and handle motif in cyan. B. A close-up view of interactions around ring-ring interface. The color coding as in A is used. The salt bridge and hydrogen bonding between Asp 170 and Arg 171 are indicated by black dashed lines. C. Alignment of seven monomers extracted from one heptamer to show side chain orientations of Asp 170 and Arg 171. This is performed in PyMOL with a core RMSD 0.20 Å². D. Presentation of the electron density map for N-terminal loop. The 2Fo-Fc electron density map is shown in blue and contoured at 1.0 σ level. The intramolecular hydrogen bonds locking the stable β-sheet structure are indicated by black dashed lines. The residues are shown according to SaClpP sequence.

**FIGURE 4.** Crystal structure of the compressed SaClpP. A. Cartoon presentation of the overall SaClpP in the compressed state. The color coding as in Fig. 3A is used. The tetradecamer is generated through symmetric operation in PyMOL to show crystal packing. The N-terminal loops are fully disordered, and no electron density could be traced. B. A close-up view of the ring-ring interface. Residues Asp 170 and Arg 171 are shown as sticks and the interaction network disappears. C. Alignment of seven monomers extracted from one heptameric ring to show the relative positions of Asp 170 and Arg 171. This is performed in PyMOL to give a core RMSD value of 0.22 Å². D. Conformational stabilization of handle helix E. The residues involved in anchoring helix E in a kinked conformation are shown as sticks with the hydrogen bonds depicted as black dashed lines.

**FIGURE 5.** Structural comparisons between the extended and compressed SaClpP. The electrostatic surface is calculated in DelPhi (54). A. Electrostatic surface presentation of the extended SaClpP tetradecamer with ~96 Å in height and ~100 Å in diameter. N-terminal loops define a 12 Å axial pore in diameter based on residue Asn 12 (bottom panel). The region of ring-ring interfaces is highlighted with a box in black dashed lines. B. Electrostatic surface presentation of the compressed SaClpP for side (top) and top (bottom) views. The compressed structure is shown as a tetradecamer in the way of crystal packing in dimensions of ~84 Å in height (excluding the N-terminal loops) and ~108 Å in diameter. The axial pore surrounded by Asp 19 is estimated to be 20 Å in diameter (bottom panel). C. Monomeric overlay of the two structures. This is performed in PyMOL with a small RMSD 0.56 Å² showing great architectural similarity. The monomer taken from the compressed structure is colored in pink, the extended structure in slate. D. Overall superimposition of the two rings from the extended (slate) and compressed (pink) structures presented in cylindrical cartoons. This is conducted in Gromacs software with a core RMSD 22.83 Å². The motif motion in the compressed SaClpP is indicated as a solid arrow compared to that in the extended tetradecamer. E. The superimposition is performed by using one monomer as reference. Side views of the alignment are provided at different angles, and the relative movements are indicated by a solid arrow.

**FIGURE 6.** Molecular dynamic simulation trajectory. A. Fluctuation of handle helix E. Superimposition of two monomers from the extended (in yellow) and compressed structures (in cyan), respectively, is performed in PyMOL using 155 atoms with a core RMSD 0.567 Å². The fluctuation range is estimated to be within an angle around 80 degrees. Residues involved in anchoring helix E in either the extended or kinked positions are shown as sticks. Hydrogen bonds are indicated with black dashed lines. B. Residue dynamics obtained by averaging atomic fluctuations over the 200 ns simulation for SaClpP monomer. The highly flexible domains including N-terminal loop (green), handle helix E (red), and helix
F (purple) are highlighted. C. Time dependence of the RMSD of the Ca atoms in the 200 ns MD simulations for the SaClpP monomer (black) and one heptameric ring of the extended tetradecamer (red). D. Structural superimposition of the active triad residues for the typical snapshots from the MD trajectory: 50 ns (cyan), 100 ns (magenta), 150 ns (orange), 200 ns (purple). Positions of His 123 and Asp 172 in the extended (green) and compressed states (red) are also shown.

FIGURE 7. **Differences in the ATPase-binding pockets of SaClpP structures.** A. ADEP1 binding in the hydrophobic pocket of EcClpP (PDB code 3MT6). The surface of EcClpP is colored in green, and ADEP1 is shown as yellow sticks. The extended channel for ADEP1 aliphatic chain binding is marked by a dashed circle. B. ADEP1 binding model in the extended SaClpP pocket, generated by an overlay on the activated state of EcClpP. The same orientation and labeling manner are used as in A. Protein surface is colored in slate. C. ADEP1 binding model in the compressed SaClpP pocket (pink) shown in the same orientation as in A.

FIGURE 8. **Proposed conformational switching between these two SaClpP states.** A. The catalytic triads and interlocking region from the substrate-binding structure of HpClpP (PDB code 2ZL2) are shown along the axial inside proteolytic chamber. The three monomers in contact with each other are colored in green, slate, and pink, respectively, and the bound peptide in magenta. B. Key residues involved in catalytic degradation and ring-ring interaction in the extended SaClpP are shown as sticks in an orientation similar to those in A. The potent motions for residues His 123 and Asp 172 in the active site, and handle helix E are indicated by arrows. C. The active site and interlocking residues as in B positioned new directions in a disordered manner in the compressed SaClpP. The handle helix E kinks and flips away. Therefore, Glu 135 is not involved in hydrogen bonding with other residues as observed in B. The substrate peptide-binding space is squeezed.

### Table 1 Data collection and refinement statistics

|                         | Compressed SaClpP | Extended SaClpP |
|-------------------------|-------------------|-----------------|
| **Data collection**     |                   |                 |
| Space group             | P6_22             | C2              |
| Cell dimensions         |                   |                 |
| a, b, c (Å)             | 121.3, 121.3, 404.4 | 168.6, 96.3, 192.6 |
| α, β, γ (°)             | 90, 90, 120       | 90, 91.4, 90    |
| Resolution (Å)          | 50.0-2.43 (2.52-2.43) * | 50.0-2.2 (2.28-2.20) * |
| No. of observations     | 699337            | 477512          |
| No. unique              | 67444             | 155453          |
| R_{sym}                 | 0.066 (0.384)     | 0.084 (0.537)   |
| I/σ(I)                  | 24.9 (2.6)        | 9.2 (1.2)       |
| Completeness (%)        | 98.9 (91.5)       | 95.3 (76.2)     |
| Redundancy              | 10.5 (4.5)        | 3.2 (1.9)       |
| **Data refinement**     |                   |                 |
| Resolution (Å)          | 20.0-2.43 (2.49-2.43) | 20.0-2.28 (2.34-2.28) |
| No. reflections         | 63145 (4046)      | 129699 (8407)   |
| R_{work}/R_{free}       | 23.9/27.2 (29.3/32.0) | 22.4/26.9 (28.2/34.4) |
| No. atoms               |                   |                 |
| Protein                 | 9334              | 20069           |
| Water                   | 150               | 686             |
| B-factors               |                   |                 |
| Mean B value            | 56.5              | 30.1            |
| R.m.s deviations in     |                   |                 |
| Bond lengths (Å)        | 0.007             | 0.007           |
| Bond angles (°)         | 0.957             | 0.993           |
| Ramachandran Plot*     |                   |                 |
| Most favoured (%)       | 97.3              | 98.3            |
| Allowed (%)             | 2.6               | 1.7             |
| Disallowed (%)          | 0.1               |                 |

*Highest resolution shell is shown in parenthesis.

*Values calculated in CCP4 suite using Procheck.
Structural switching of Staphylococcus Aureus CLPP: a key to understanding protease dynamics
Jie Zhang, Fei Ye, Lefu Lan, Hualiang Jiang, Cheng Luo and Cai-Guang Yang

J. Biol. Chem. published online September 7, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.277848

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/09/07/M111.277848.DC1