Protein Structures

DOI: 10.1002/anie.201100666

A Conformational Switch Underlies ClpP Protease Function**

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The barrel-shaped serine protease ClpP degrades misfolded, damaged, and regulatory proteins. Substrate proteins enter the ClpP barrel through the two axial pores, but it is unclear how the peptide products exit the barrel. Here we report the structure of ClpP from *Staphylococcus aureus*, which reveals a previously unobserved compressed state of the barrel. A conformational switch in the active center "handle region" results in closure of the active sites and opening of equatorial pores. Conserved residues in the handle region underlie the conformational switch and are functionally essential although they are not part of the active sites. These results are consistent with processive cycling of ClpP between an extended state with open active sites and closed equatorial pores, and a compressed state with closed active sites and open pores for product release.

Energy-dependent proteases control quality and quantity of cellular proteins by degrading those that are misfolded, damaged, or regulatory and thus short-lived.[1] Proteases of this family include the eukaryotic 26S proteasome and the bacterial caseinolytic protease (Clp).[2a,b] Clp enzymes contain the barrel-shaped serine protease ClpP that degrades the substrate protein, and an AAA+ ATPase such as ClpX or ClpA that selects and unfolds the substrate and translocates it into the ClpP barrel through its axial pores.[3a,b] ClpP processively digests the translocated polypeptide into peptide fragments of six to eight residues.[4] Crystal structures of ClpP from various species are highly similar and reveal two stacked heptameric rings, which confine a large chamber containing 14 active sites.[5] ClpP is a serine protease with canonical catalytic triads that each comprise a serine, histidine, and aspartate residue. Structures of ClpP bound to an inhibitor[6] or a peptide[7] revealed that the active center region is flexible and flanked by a mobile "handle region" that is functionally important.[8,9]

The key open question on the ClpP mechanism is how the peptide products are released from the active sites and how they exit from the barrel chamber. One model proposes that the peptides diffuse through the axial pores that are also used for substrate entry,[10a,b] but this model is inconsistent with published data[9] and postulates transient release of the ATPases from ClpP and thus seems incompatible with a processive function.[11] A second model proposes that the two heptameric rings dissociate transiently, but there is no evidence for this. A third model proposes that peptides are released through "equatorial" pores that transiently form on the side of the barrel.[8,9,12] However, such pores have not been observed and their formation would require major conformational changes that seem difficult without barrel dissociation. Here we report the crystal structure of ClpP from *Staphylococcus aureus* (*Sa*ClpP), which reveals the predicted equatorial pores and the conformational changes required for their formation without barrel dissociation.

*Sa*ClpP was purified and crystallized, and its structure determined by molecular replacement using ClpP from *Streptococcus pneumoniae* (*Sp*ClpP, 1Y7O)[8] as a search model (see Table 1 and the Supporting Information). Like ClpP from other species,[5] *Sa*ClpP consists of two heptameric rings that form a tetradecameric barrel (Figure 1). However, compared to all other ClpP structures, the barrel is compressed by roughly 10 Å along the axial direction, and the ring–ring interface contains 14 equatorial side pores that can be up to 6 Å in diameter, depending on the side-chain conformations (Figure 2). The side pores are lined with conserved hydrophobic residues and apparently represent the long-sought exit route for peptide products from the barrel chamber.[8,9] The compressed barrel reflects a different conformational state rather than a species-specific fold, since *Sa*ClpP shares 73% identical residues with *Sp*ClpP, and is highly conserved throughout species.

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[**] We thank Alan Cheung, Dirk Kostrewa, and other members of the Cramer laboratory for help. S.G. was supported by the Elitenetzwerk Bayern graduate program NanoBioTechnology. P.C. was supported by the Deutsche Forschungsgemeinschaft, SFB646, SFB TR5, NIM, the Jung-Stiftung, and the Fonds der Chemischen Industrie. T.B. was supported by the German National Academic Foundation and by an EXIST technology transfer grant from the Federal Ministry of Economics and Technology (BMWi). S.A.S. was supported by the Deutsche Forschungsgemeinschaft (Emmy Noether program), SFB749, and CIPSM. We thank J. Basquin and K. Saldaña from the crystallization facility of the MPI of Biochemistry, Martinsried, and Malte Gersch for helpful discussions. This work was conducted in part at the Swiss Light Source at the Paul Scherrer Institute, Villigen, Switzerland. We thank the Protein Structure Factory at BESSY, Berlin.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201100666.

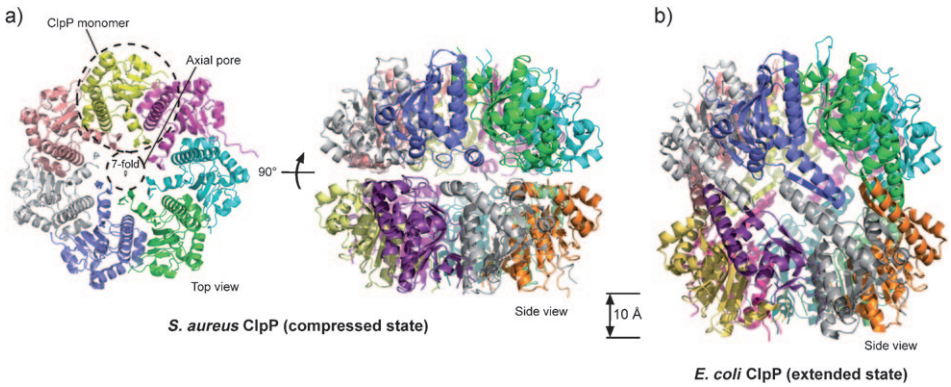

Figure 1. *S. aureus* ClpP adopts a compressed state. a) Two views of a ribbon model of the crystal structure of *S. aureus* ClpP (*Sa*ClpP) in the previously unobserved compressed state. The enzyme consists of two heptameric rings (only one depicted in top view), with a sevenfold symmetry axis in the center of the axial pore. b) Extended state of ClpP observed in previous structures. Depicted is the *E. coli* ClpP structure (*Ec*ClpP, PDB code 2FZS), which is extended by about 10 Å along the axial direction.

Table 1: Diffraction data and refinement statistics for *Sa*ClpP.

Data collection	
space group	$P2_12_12$
unit cell axes [Å]	172.1, 178.0, 100.3
wavelength [Å]	1.0000
resolution range [Å]	100–2.55 (2.65–2.55)[a]
unique reflections	100837 (10859)
completeness [%]	99.9 (99.9)
redundancy	10.4 (10.9)
mosaicity [°]	0.17
R_{sym} [%]	6.8 (78.0)
$I/\sigma(I)$	25.5 (4.9)
Structure refinement	
nonhydrogen atoms	20407
RMSD[b] bonds [Å]	0.010
RMSD[b] angles [°]	1.15
R_{cryst} [%]	20.8
R_{free} [%]	22.6
preferred residues[c] [%]	96.4
allowed residues[c] [%]	3.6

[a] Values in parentheses are for highest-resolution shell. [b] Root mean square deviation. [c] Ramachandran plot statistics from PROCHECK.[14]

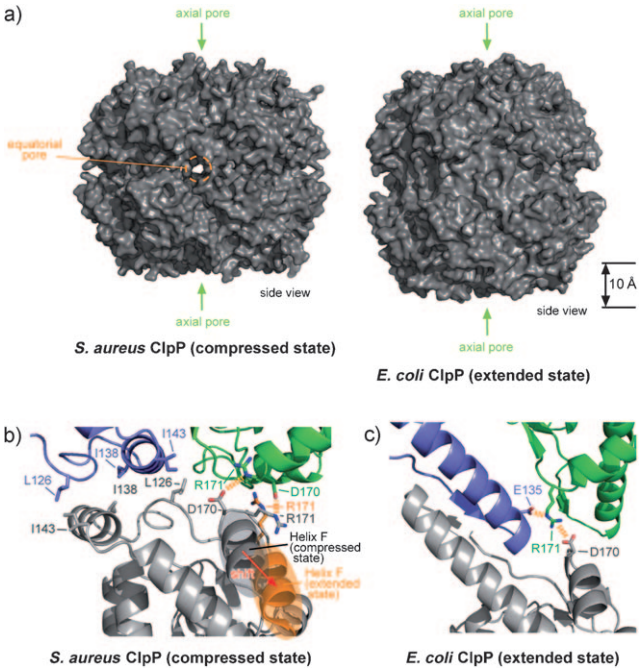

Figure 2. Transition in the ClpP ring–ring interface and formation of equatorial pores. a) Surface representation of *Sa*ClpP (compressed state with an equatorial pore indicated, left) and *Ec*ClpP (extended state without equatorial pores, right). b) Close-up view of the ring–ring interface in the compressed state observed for *Sa*ClpP. Stabilizing hydrophobic residues and salt bridges between D170 and R171 of adjacent subunits are depicted. The alternative location of helix F in the extended state is indicated (orange). c) Rearranged ring–ring interface salt bridges in the extended state (compare with the view in b).

The structure of the ClpP monomer is very similar to other ClpP structures, except for a major difference in the handle region (Figure 3). Whereas the handle region in all known structures consists of a straight or partly disordered αE helix and an adjacent β6 strand, αE in the *Sa*ClpP structure is kinked and β6 is disordered (Figure 3b). This strong structural difference accounts for the compression of the ClpP barrel and the opening of the side pores. Superposition of the *Sa*ClpP structure with the *H. pylori* ClpP–substrate complex (*Hp*ClpP, 2L2) revealed that the kinked handle helix clashes with the substrate (Figure 3c), showing that the kinked ClpP structure represents an inactive state. The catalytic triad is also in a nonfunctional arrangement (Figure 3d). The kinked helix shows high crystallographic B-factors (Figure 3a), consistent with mobility and the different conformations observed by NMR spectroscopy[9] and predicted from biochemical data and calculations.[12] This suggests that a conformational switch exists that converts the previously observed ClpP state to the new compressed state observed here. The compressed state is distinct from a previously described "compact state" of ClpP,[8,12] which is close to the extended state, does not show the kink in helix αE, and reveals only a minor shift of helix αF (Figure 4). However, the compact state may be an intermediary state at the beginning of the transition from the extended to the compressed state.

Structural comparisons suggest that conserved residues form a hinge that underlies a functionally required conformational switch in the handle region. To test this, we mutated

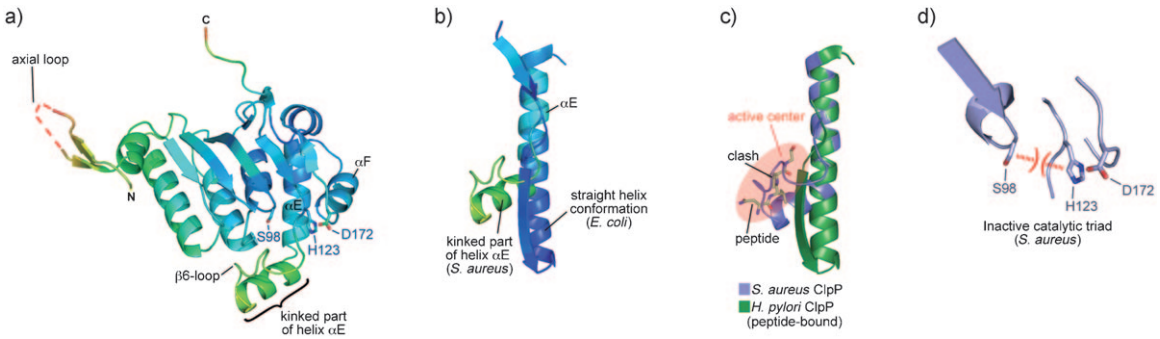

Figure 3. Active sites are closed in the compressed ClpP state. a) *Sa*ClpP monomer is shown in rainbow colors corresponding to crystallographic B-factors ranging from blue (low) to red (high). Secondary structure elements, the axial loop, the handle domain, and residues of the catalytic triad are labeled. b) Superposition of the kinked αE helix in the compressed state (*Sa*ClpP) with the straight αE helix in the extended state (*Ec*ClpP, PDB code 2FZS, dark blue). c) The kinked helix **α**E closes the active site in the compressed state (*Sa*ClpP). The kinked part of helix αE in *Sa*ClpP (light blue) shows a clash with the peptide bound to the active center of *Hp*ClpP, which is in the extended state (green, 2ZL2). d) *Sa*ClpP shows an inactive conformation of the catalytic triad. Catalytic residues S98, H123, and D172 are indicated.

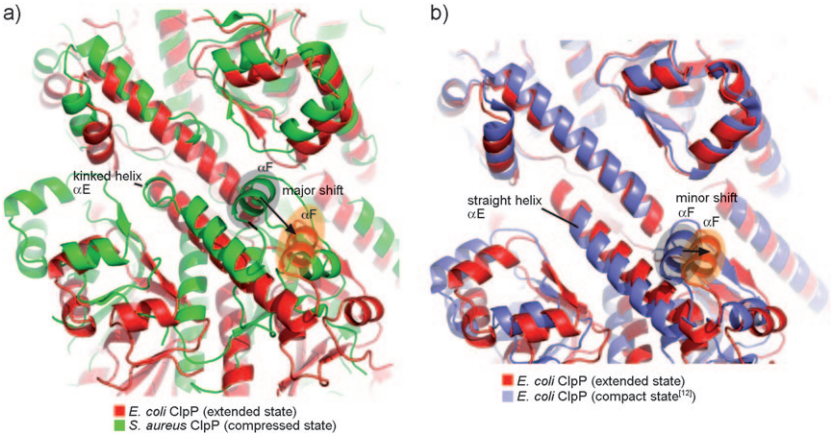

Figure 4. The ClpP compressed state is distinct from a previously described compact state. a) Superposition of *Sa*ClpP (compressed state) with *Ec*ClpP in the extended state (2FZS) reveals kinking of helix αE and a major shift in helix αF. b) Superposition of *Sa*ClpP (compressed state) with *Ec*ClpP in the compact state (3HLN)[12] reveals no kinking of helix αE and only a minor shift in helix αF.

hinge residues that were not part of the catalytic center, and analyzed the effect of these mutations on ClpP function, as monitored by cleavage of a model substrate (see the Supporting Information). In these assays, wild-type ClpP was active ($K_m = 580$ μM, $k_{cat} = 0.093\ s^{-1}$) and mutation of the catalytic residue S98 to alanine abolished activity (Figure 5), providing a positive and a negative control, respectively. Two invariant glycine residues, G127 and G131, along with G128 form part of the hinge, and their mutation to alanine abolished activity (Figure 5b). Residue E135 forms a salt bridge with R171 when the handle helix is straight, but not when the handle helix is kinked (Figure 5a), suggesting that the salt bridge stabilizes the active open state. Consistent with this model, mutation of E135 to alanine or arginine also abolished activity (Figure 5b). Similarly, L144 adopts different positions in the two states and forms contacts that apparently stabilize the kinked state (Figure 5a). Consistent with this proposal, mutation of L144 to an isosteric hydrophobic methionine residue resulted in partially retained activity (L144M, $K_m = 523$ μM, $k_{cat} = 0.003\ s^{-1}$), whereas mutation to glycine, glutamate, or arginine essentially abolished activity (Figure 5b). These results are consistent with a functionally essential switch that interconverts the two observed ClpP conformations.

These results pose the question how such a large-scale conformational change can be accommodated without ring–ring dissociation, in particular since the two heptameric rings are rotated with respect to each other by eight degrees around the axial direction. A detailed analysis of changes in the ring–ring interface revealed that helix αF shifts up to 9 Å, and that residues D170 and R171 in a loop adjacent to this helix form salt bridges with their counterparts in the opposite ring (Figure 2b). These salt bridges are rearranged but maintained in the active, extended state of ClpP (Figure 2c),[6] and are essential for stabilization of the ring–ring interface.[8] These results indicate that the ring–ring interface is stabilized by these two contacts close to the αF helices in both rings. Consistent with these results, disruption of the handle domain does not lead to ring–ring dissociation,[8] and an intact ClpP tetradecamer with a partially disordered handle domain was observed for *M. tuberculosis* ClpP.[13]

Our results and published data converge on a dynamic two-state model of processive ClpP protease function. According to this model, Clp-mediated protein degradation occurs in repetitive cycles that comprise three steps. First, the ClpX/A ATPases unfold the target protein and translocate it through the ClpP axial pores into the ClpP barrel chamber. Second, the protein chain is cleaved by the ClpP protease active sites, generating peptide fragments. Third, a conformational switch in the handle region and axial ring–ring rotation close the active sites and open equatorial side pores, allowing product release and exit. This reaction cycle explains the processive nature of protein degradation by ClpP, as it

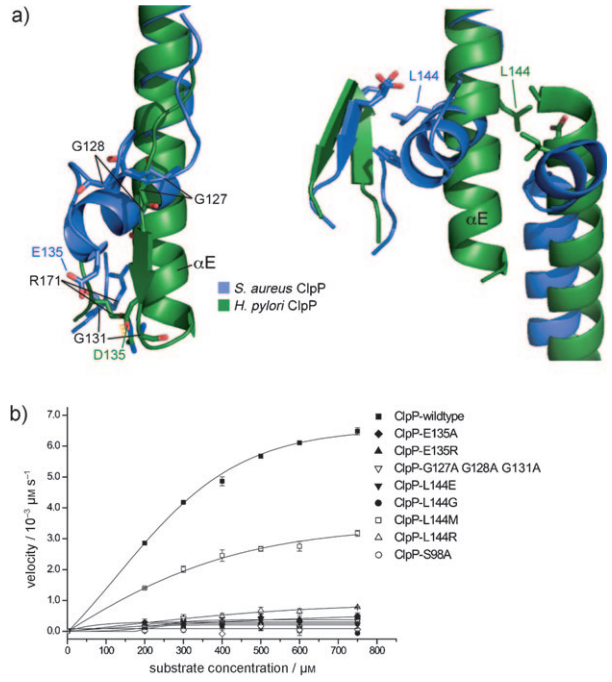

Figure 5. A conformational switch underlies ClpP function. a) Location of ClpP residues involved in the conformational switch of the handle region. Residues G127, G128, and G131 are indicated in the compressed *Sa*ClpP (blue) and the extended *Hp*ClpP (green, 2ZL2). Residues R171 and E135 form a salt bridge in the extended state, but not in the compressed state. Residue L144 forms contacts with different residues in both states. b) Michaelis–Menten kinetics of ClpP mutants. ClpP wild-type enzyme is shown as a positive control, ClpP variant S98A as a negative control.

requires neither ring–ring nor protease–ATPase dissociation. To illustrate the key conversion of the previously reported extended state of the ClpP barrel[7] to the compressed state reported here for *Sa*ClpP, we prepared an animation (see the Supporting Information).

Experimental Section

Details for production and purification of the recombinant protein are given in the Supporting Information. In short, the *E. coli* BL21 cells producing recombinant *S. aureus* ClpP were lyzed and after treatment with 0.1% polyethyleneimine (pH 8.0) the supernatant was subjected to anion exchange chromatography (HiTrap Q HP, GE Healthcare). ClpP was subsequently purified on StrepTactin Superflow beads (IBA), and crystals were grown in hanging drops at 20°C using as reservoir solution 1.8 M ammonium sulfate and 100 mM sodium acetate (pH 4.5). The structure was solved by molecular replacement with AMORE.

Mutagenesis was carried out with the QuikChange II site-directed mutagenesis kit (Stratagene). Michaelis–Menten kinetics of the ClpP variants were determined by the cleavage of the fluorogenic model substrate *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC) and release of AMC.

Coordinates and structure factors for the *S. aureus* ClpP structure have been deposited in the Protein Data Bank (accession code 3QWD).

Received: January 26, 2011
Published online: May 4, 2011

Keywords: ClpP heat shock protein · conformational switch · protease complex · protein structures · virulence regulation

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