Cryro-EM structure of the ClpXP protein degradation machinery

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The ClpXP machinery is a two-component protease complex that performs targeted protein degradation in bacteria and mitochondria. The complex consists of the AAA+ chaperone ClpX and the peptidase ClpP. The hexameric ClpX utilizes the energy of ATP binding and hydrolysis to engage, unfold and translocate substrates into the catalytic chamber of tetradecameric ClpP, where they are degraded. Formation of the complex involves a symmetry mismatch, because hexameric AAA+ rings bind axially to the opposing stacked heptameric rings of the tetradecameric ClpP. Here we present the cryo-EM structure of ClpXP from Listeria monocytogenes. We unravel the heptamer-hexamer binding interface and provide novel insight into the ClpX-ClpP cross-talk and activation mechanism. Comparison with available crystal structures of ClpP and ClpX in different states allows us to understand important aspects of the complex mode of action of ClpXP and provides a structural framework for future pharmaceutical applications.

Caspolytic protease (ClpP) represents a major proteolytic protein in prokaryotes and in organelles of eukaryotes that is involved in protein homeostasis, bacterial pathogenesis and cancer progression. ClpP is highly conserved and is essential for virulence and regulation of stress responses in several pathogenic bacteria; thus it is considered to be a promising therapeutic target for novel antibiotics. ClpP associates with diverse ATP-dependent AAA+ chaperones such as ClpX, ClpC and ClpA to form a complex for the recognition, unfolding and digestion of substrate proteins. To date, a large fraction of research has been dedicated to functionally exploit ClpP and its cognate chaperones, foremost ClpX, in terms of their enzymatic activity, individual structures and conformational control.

Previous low-resolution EM studies of ClpXP and ClpAP from Escherichia coli have revealed that up to two hexameric ClpX chaperones bind to a ClpP tetradecameric barrel. The barrel consists of two stacked heptameric rings, forming a degradation chamber with 14 proteolytic sites. Each ClpX subunit consists of an N-terminal zinc binding domain (ZBD) and a C-terminal AAA+ domain. The ZBDS at the periphery of ClpX are responsible for recognition and engagement of several substrates. ClpX hydrolyzes ATP to unfold the target substrates and translocate the unfolded polypeptides through a central pore into the proteolytic chamber of the ClpP barrel (reviewed in ref. 10).

Early on, the hexamer-heptamer ClpX-ClpP interface fascinated researchers, and several studies characterize the role of putative interaction motifs have led to models explaining the symmetry mismatch and functional interaction between the two proteins. Sequence alignments and mutational studies of AAA+ chaperones identified loops in ClpX that interact with the hydrophobic clefts on the periphery of ClpP. These loops contain the highly conserved (I/L/V)-G-(F/L) motif and are essential for complex formation.

More recently, cyclic acyldespipeptides (ADEPs), a novel class of anti-bacterial compounds, have been identified to bind to the same peripheral hydrophobic clefts on ClpP and to induce the opening of the axial pores of ClpP. They stabilize ClpP in an ‘open’ activated state in the absence of the chaperone, leading to unregulated proteolysis of substrates and, finally, to cell death. This suggests that the protruding loops in ClpX that contain the (I/L/V)-G-(F/L) motif, or IGF loops, are sufficient to activate ClpP. It has also been speculated that this activation involves the opening of the axial pore to allow translocation of the substrate into the proteolytic chamber of ClpP. However, owing to the lack of high-resolution structures, a detailed understanding of the interaction between ClpX and ClpP is missing.

Contacts between the pore-2 loops of ClpX and the N termini of ClpP represent a second set of well-characterized interactions between ClpX and ClpP, which are, however, more dynamic and dependent on the nucleotide state of ClpX. A crucial function of the ClpP N termini is to gate the entrance of the proteolytic chamber. Despite these detailed biochemical insights, a high-resolution structure of the whole proteolytic complex is lacking, thereby limiting our understanding of this important protein degradation machinery. Here we present a 4-Å cryo-EM structure of ClpXP1–2 from L. monocytogenes.

Results

Cryro-EM structure of ClpXP1–2. In order to obtain a ClpXP complex that is suitable for structural studies, we used the ClpP1–2 from L. monocytogenes. In contrast to other bacteria, L. monocytogenes encodes two ClpP isoforms, LmClpP1 and LmClpP2, which can assemble into hetero-oligomers composed of two homoeptameric rings. Recent studies have revealed that ClpP1–2 has a higher affinity to ClpX compared with the more-conserved ClpP2 homomonomer, suggesting a superior stability of the hetero-oligomer. Because ClpP1–2 might cleave ClpX to a small extent during sample preparation, we mutated one residue of the catalytic triad (S98A) in both ClpP isoforms. Furthermore, we mutated the nucleotide binding site of ClpX (E183Q) to allow ATP binding but prevent hydrolysis, which results in a tighter binding to ClpP.

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We formed a complex of ClpX and ClpP1−2 and obtained a large fraction of ClpXP1−2 dimers (ClpP1−ClpP2−ClpX−ClpP1−ClpP2) that were in equilibrium with ClpXP1−2 monomers (Supplementary Fig. 1a–c). It has been demonstrated previously that two ClpP or ClpA hexamers can bind to one ClpP barrel from both sites, resulting in a ClpXP−ClpP or ClpA−ClpP−ClpA complex. However, ClpXP1−2 dimers (Supplementary Fig. 1a–d) have not yet been described, to our knowledge. We therefore first concentrated our structural analysis on these intriguing dimers and determined their structure by means of cryo-EM and single-particle analysis using crYOLO and SPHIRE (Table 1, Fig. 1a,b and Supplementary Fig. 1e–g). Although the intrinsic flexibility of the complexes did not allow the determination of a high-resolution structure (Supplementary Video 1 and Supplementary Fig. 1e), the fitting of the crystal structure of ClpX into the cryo-EM density suggests that the flexible N-terminal ZBDs of ClpX mediate the interaction between two ClpX hexamers (Fig. 1c). Whereas ZBD-deleted ClpX still associated with ClpP to a small extent, ClpX dimerization was completely abolished, thus supporting our structural data (Supplementary Fig. 1a).

The ZBDs are involved in substrate binding and cofactor recognition and were shown to dimerize when expressed as single domain. On the basis of these results, it has previously been proposed that the ZBDs of neighboring subunits within a single ClpX hexamer dimerize, resulting in a trimer-of-dimer model. In this model, the ZBD dimers interact with the adjacent dimers, creating a ring structure that is aligned with the central channel of ClpX. The structure of the ClpXP1−2 dimer, however, reveals that the ZBDs do not form rings but arrange in a flexible half-cone spiral, with the first and last ZBD dimers positioned directly above or at the rim of the axial pore entry of the upper and lower ClpX hexamer, respectively (Fig. 1c and Supplementary Fig. 1e). The ZBDs apparently interact with the ZBDs of the other ClpX or by ZBD dimers that interact with dimers of the other ClpX. On the basis of these results and the fact that the ZBDs are flexible and not resolved in the crystal structure of ClpX, we propose that ZBD dimers form stable structures only at the interface between two oppositely positioned ClpX hexamers (Fig. 1d).

To obtain a cryo-EM structure at a higher resolution, we focused the structural analysis on one ClpXP1−2 subunit in the dimer and solved its structure using the same data set (Table 1, Fig. 2a–d and Supplementary Fig. 2). The final cryo-EM reconstruction has an average resolution of 3.6–4 Å for ClpXP1−2 and 6–7 Å for ClpX (Supplementary Fig. 2e–g). The overall lower resolution of ClpX indicates that the chaperone is intrinsically more flexible and heterogeneous than the ClpP barrel in the ClpXP1−2 complex. To build a complete atomic model of ClpXP1−2, we fitted a homology model of ClpX and the available crystal structure of ClpP1−2 (PDB 4RYF) into the cryo-EM density and refined the model using molecular dynamics flexible fitting.

The structure of ClpXP1−2 reveals that ClpP1 forms the upper homopeptamer of the ClpP barrel, whereas ClpP2 sits below and interacts with ClpX (Fig. 2c–h). Our cryo-EM structure is consistent with previous binding studies on L. monocytogenes and Mycobacterium tuberculosis ClpP proteases, showing ClpX−ClpP1−2 interactions exclusively via the ClpP2 ring surface.

Notably, the ClpX hexamer is not centrally aligned but tilted ~11° toward ClpP2. The structure of ClpXP1−2 is almost identical to that of the available crystal structure of apo-ClpP1−2 (PDB 4RYF), indicating that the binding of ClpX does not induce large conformational changes in ClpXP1−2. In contrast, interaction with ClpP1−2 has an effect on the overall conformation of ClpX. Whereas the crystal structure of E. coli ClpX shows the ATPase domains in a dimer-of-trimers arrangement, our structure shows that upon ClpP1−2 binding, these domains become more regularly arranged and are related by pseudo-six-fold symmetry. Unlike recent substrate-bound AAA+ structures that show a spiral-staircase arrangement with one ‘seam’ subunit moderately displaced from the pore, all neighboring AAA+ domains of ClpX pack closely with each other. The resolution at the nucleotide pocket is not high enough to visualize nucleotides, but the structure reveals that all six ClpX protomers are in the ‘loadable’ (L) conformation (Supplementary Fig. 3). This conformation is in contrast to that of ClpX with the E183Q mutation in its apo state, in which two subunits are in the L conformation, and four are in the ‘unloadable’ (U) conformation (Supplementary Fig. 3). In the L state, the arrangement of the small and large AAA+ modules of ClpX is very similar to the structure of ClpXP1−2, but the conformational change of the nucleotide pocket is not high enough to visualize the nucleotides.

Table 1 | Cryo-EM data collection, refinement and validation statistics

| LmClpX1−2 dimer | LmClpXP1−2 EMD-10162, (PDB 6SFX, PDB 6SFW) |
|-----------------|---------------------------------------------|

| Data collection and processing | Magnification | 112,807 | 112,807 |
|-------------------------------|---------------|--------|--------|
| Voltage (kV)                  | 300           | 300    |        |
| Electron exposure (e−/Å²)     | 114           | 114    |        |
| Defocus range (μm)            | 0.5 to −3.0   | −0.5 to −3.0 |
| Pixel size (Å)                | 1.1           | 1.1    |        |
| Symmetry imposed              | C1            | C1     |        |
| Initial particle images (no.) | 273,300       | 613,322 | |
| Final particle images (no.)   | 143,901       | 383,927 | |
| Map resolution (Å)            | 13            | 4      |        |
| FSC threshold                 | 0.143         | 0.143  |        |
| Map resolution range (Å)      | −3.2 to 10    | 6SF X  | 6SF W  |

| Refinement                   | Initial model used (PDB code) | − | 4RYF |
|-------------------------------|-------------------------------|---|------|
| Model resolution (Å)          | −                             | 2.8  | −     |
| Model resolution range (Å)    | −                             | −    | −     |
| Map sharpening B factor (Å²)  | −                             | −214 | −240  |

| Model composition            | Nonhydrogen atoms | 20,196 | 15,225 |
|-------------------------------|-------------------|--------|--------|
| Protein residues              | −                  | 2,602  | 1,955  |
| B factors (Å²)                | −                  | 100.8  | 187.87 |
| R.m.s. deviations             | −                  | −      | −      |
| Bond lengths (Å)              | −                  | 0.012  | 0.018  |
| Bond angles (°)               | −                  | 1.233  | 1.978  |

| Validation                    | MolProbity score | −2.34 | 2.30  |
|-------------------------------|------------------|-------|-------|
| Clashscore                    | −22.88           | 22.43 |
| Poor rotamers (%)             | −0.18            | 0.20  |

| Ramachandran plot             | Favored (%)      | −92.04| 92.9  |
|-------------------------------|------------------|-------|-------|
| Allowed (%)                   | −7.65            | 6.40  |
| Disallowed (%)                | −0.31            | 0.70  |
domains results in an open binding cleft, to which the nucleotide can bind. In the U state, this site is blocked. A dynamic interconversion between L and U conformations is required to couple ATP hydrolysis by ClpX to mechanical work. However, the arrangement is not a direct consequence of the bound nucleotide or the presence of specific mutations28.

Fig. 1 | LmClpXP1−2 forms flexible dimers via the ZBDs. a, A representative low-dose cryo-EM micrograph of the ClpXP1−2 dimer from L. monocytogenes. Some particles are highlighted with ovals. b, Representative reference-free 2D class averages. Arrows indicate additional densities corresponding to ZBDs at the interface between two ClpX hexamers. c, Ribbon model of ClpP1 (yellow), ClpP2 (green) and ClpX (orange) superimposed with the cryo-EM density map of the ClpXP1−2 dimer (white and transparent). Upper inset, the complex as a slice at the position of the axial pore entry of the upper ClpXP1−2 complex. ClpX and ClpX-ZBD densities are colored magenta and gray transparent, respectively. The arrow indicates the spiral arrangement of the ZBD domains. Lower inset, four copies of ZBD dimers (PDB 1OVX) placed into the cryo-EM density at the interface between the ClpX hexamers. The low-resolution density did not allow automated rigid-body fitting; therefore, the dimers were placed manually and interconnected, as proposed in ref. 26. d, Cartoon depicting ClpXP1−2 dimerization via the ZBD domains of two opposing ClpX hexamers. Arrows indicate the flexibility of the complex.

Fig. 2 | Cryo-EM structure of the ClpXP1−2 protein degradation machinery. a–d, Cryo-EM density of ClpXP1−2 shown from the top (a), bottom (b) and sides (c,d). ClpP1 and ClpP2 subunits are colored in tan and orange and dark and light green, respectively. ClpP2 subunit J is highlighted in mint green. Note that this is the only ClpP2 subunit not interacting with ClpX via an IGF loop. Each subunit of ClpX (O–S) has been assigned a color; this color code is maintained throughout all figures. e,f, Molecular model of ClpXP. The hydrophobic pockets of ClpP2, each spanning two ClpP2 subunits, are shown as surface. The IGF interaction loops are highlighted in red. g,h, Cartoons depicting how the ClpX hexamer interacts with the ClpP2 heptamer via the six IGF loops. Note the extended conformation of the IGF loop of ClpX subunit Q.
To further examine the interaction between ClpP1−2 and ClpX, we used hydrogen/deuterium-exchange MS (HDX-MS) to monitor the accessibility of residues at the interface. In line with our structural observations, complex formation between ClpP1−2 and ClpX only changes the accessibility of residues of ClpP2 and ClpP2, but not of ClpP1 (Supplementary Fig. 4). This finding not only corroborates that ClpX solely interacts with the ClpP2 isoform but also indicates that ClpX binding does not induce major allosteric conformational changes in the ClpP1 heptamer.

Symmetry mismatch of IGF loop interaction. The most interesting part of the structure is the interface between ClpP2 and ClpX, which involves a C6−C7 symmetry mismatch. As predicted by biochemical studies37−40, it is mediated mainly by the flexible IGF loops of ClpP2 interacting with hydrophobic grooves in ClpP2 (Fig. 2c,d and Supplementary Fig. 5a). The tilted arrangement of ClpP2 results in part of the loops interacting more strongly with ClpP2 than others (Fig. 3a).

The large domains of the respective ClpX subunits from which the loops protrude are positioned directly below the deep hydrophobic grooves of ClpP2, which are formed at the interface of two subunits. This arrangement allows a direct interaction of the IGF loops with the opposing grooves. The hydrophobic grooves of ClpP are arranged in a circular manner with seven-fold symmetry, and the positions of the ClpX IGF loops in the complex perfectly match this arrangement. Notably, both rings show similar diameters (Fig. 3b,c), except the IGF ring remains open at the position of the seventh, free hydrophobic groove.

Five of the six IGF loops (subunits O, P, R, S, T) display an over-all similar arrangement. Due to the symmetry mismatch, the large domain of the sixth subunit (subunit Q), is positioned between two hydrophobic grooves. The respective IGF loop, however, still interacts with one of the opposing grooves by adopting an ‘extended’ conformation (Fig. 2c−d). The other groove stays empty. Although the distance between the IGF loop and the ‘left’ and ‘right’ ClpP hydrophobic grooves are similar, we only obtained a high-resolution structure with the IGF loop binding exclusively to the left binding pocket.

To support our structural findings, we performed HDX-MS measurements and mutational studies. Upon complex formation, deuterium uptake of the IGF loop was strongly reduced (Fig. 4 and Supplementary Fig. 4), and mutations in the IGF loops of ClpX and the hydrophobic grooves of ClpP2 resulted in impaired complex formation (Supplementary Fig. 6). This finding is in line with our ClpXP1−2 structure that demonstrates that the interaction between the IGF loops and the hydrophobic grooves is crucial for complex formation and function.

Taken together, tilting of the ClpX ring and stretching of one of the IGF loops is sufficient for the hexameric ClpX to adapt to the seven-fold symmetry of the heptameric ClpP, leaving one of the binding pockets (Fig. 2g,h). Due to multivalence, this results in strong but flexible binding, which is likely necessary to accommodate the different conformations of ClpX protomers during ATP hydrolysis and substrate processing37−40.

N termini of ClpP2 and pore-2 loops of ClpX regulate the entry portal. ClpX is not only tilted but also laterally shifted respective to ClpP2 (Fig. 3a,c). Such an arrangement has also been described for other complexes that display a symmetry mismatch37−39. In the case of ClpXP1−2, this arrangement results in misalignment of the central channels of ClpP and ClpX, creating a twisted translocation channel with a constrictive site at the interface between ClpP2 and ClpX.
Different conformations of the ClpP N-terminal loops have been previously identified in crystal structures of apo and ADEP-bound ClpPs\(^{11,41,42}\). In the \textit{E. coli} apo ClpP structure, the N termini on the apical side of the ClpP barrel are in the ‘down’ conformation, opening one axial pore of the barrel. On the basal side, six of the N termini are in the ‘up’ conformation, with the loops moving out of the axial pore, thereby covering and closing it. It was speculated that the six ClpP N termini in the ‘down’ conformation would open to match the six-fold symmetry of ClpX, and the seventh non-interacting N terminus would stay in the ‘down’ conformation upon binding to the chaperone. However, in the ADEP-bound structure of \textit{E. coli} ClpP, all loops point upward, whereas in a \textit{Bacillus subtilis} ADEP-bound ClpP structure, they are not resolved, having made general conclusions difficult so far\(^{11,41}\).

In our cryo-EM structure, residues 6–17 are not resolved, but the rest of the density reveals that all seven N termini of ClpP2 (the apical side of the barrel facing the chaperone) adopt the ‘up’ conformation, thus resolving the uncertainty about their positioning and the accessibility of the pore (Supplementary Fig. 7). The cryo-EM structure demonstrates that the interaction site between the ClpP2 N termini and the ClpX pore-2 loops is not shielded and freely solvent accessible. Additionally, the N termini undergo a conformational change upon complex formation and adopt the ‘up’ conformation, by which the protein backbone likely gets more solvent exposed and/or flexible. In line with this notion, deuteration of the ClpP2 N terminus increased after complex formation (Fig. 4 and Supplementary Fig. 4). This observation is also supported by previously reported synchrotron hydroxyl radical footprinting data showing that ClpA binding enhanced the modification rate of an N-terminal peptide of ClpP, pointing toward a higher solvent accessibility\(^{41}\).

The C terminus of ClpP2 shields the hydrophobic groove prior to ClpX binding. The C termini of the ClpP2 show two conformations in our structure: a compact conformation that blocks the hydrophobic groove when it does not accommodate an IGF loop and an extended conformation enlarging the groove when occupied by an IGF loop (Fig. 5a). Because the residues of the C terminus are not conserved (Supplementary Fig. 9) and the conformational change is not transmitted to the rest of the protein, an allosteric regulation is unlikely. The C termini probably shield the hydrophobic grooves when ClpX is not bound and thereby prevent the interaction with other hydrophobic molecules and increase the stability of the protein in a hydrophilic environment.

To probe this, we deleted the last three to six amino acids of ClpP2. ClpP1–2\(^{41,46}\) precipitated during purification, suggesting that a certain length of the C terminus is important to protect the hydrophobic groove and facilitate protein stability. ClpP2 mutants bearing three to five amino acid deletions were, however, soluble and exhibited a similar peptidolytic activity as the wild-type complex (Fig. 5b). Interestingly, in protease assays requiring the binding of ClpP2, N terminus increased after complex formation (Fig. 4 and Supplementary Fig. 5a). We interpret this result such that when the C termini are shorter more complexes are formed because ClpX can more easily access the hydrophobic grooves via the IGF loops. When ClpP2 is not bound and thereby prevent the interaction with other hydrophobic molecules and increase the stability of the protein in a hydrophilic environment.

The C termini of ClpP2 were shorter in length (Supplementary Fig. 9).

\textbf{ClpP activation mechanism by ClpX.} Previous crystal structures of ClpP in its apo form, that is, without ClpX or compound bound, revealed three different conformational states of the protein: ‘compressed’, ‘compact’ and ‘extended’\(^{44–48}\) (Fig. 6). The catalytic triad of the peptidase is only intact in the extended state, suggesting that this is the only active state. ADEPs, which bind to the same site on ClpP as the IGF loops, can induce the transition from the

\textbf{Fig. 4 | HDX-MS analysis of ClpXP1–2 complex formation.} a, Difference in relative deuterium uptake after 10 s of exposure is mapped on the structure of ClpXP1–2 (left), ClpP2 monomer (top right) and ClpX monomer (bottom right). Increased deuterium uptake upon complex formation is shown in red, and decreased deuterium uptake is shown in blue. Dark gray represents no coverage. The MS data are available online as source data. b, HDX kinetics of exemplary peptides in the N terminus of ClpP2 (top) and in the IGF loop of ClpP2 (bottom). Solid lines and filled circles represent the ClpXP1–2 complex; dashed lines and empty circles represent ClpP1–2 or ClpX. Two independent replicates are shown; lines denote the mean.

ClpX (Fig. 3d). At this position, the N-terminal loops of ClpP2 and pore-2 loops of ClpX interact with each other. These interactions are expected to be even more dynamic than the flexible contacts mediated by the IGF loops and coupled to ATP hydrolysis\(^{12,14,40}\). The densities corresponding to the N-terminal loops of ClpP2 and pore-2 loops of ClpX are weak, indicating a higher degree of flexibility in this region of the complex (Supplementary Figs. 7 and 8).
compressed to the extended conformation\(^{15}\). Additionally, an \(-90^\circ\) rotation of Tyr63 in the hydrophobic pocket resulted in the widening of the axial pore by 10–15 Å. A mutation of this residue to alanine has the same effect\(^{16}\). This ‘open’ extended conformation of ClpP deregulates the protein. Instead of only processing short peptides of five to six residues, it is now capable to degrade large unfolded polypeptides that otherwise could not be processed in the absence of the chaperone\(^ {12,43,50}\) (Fig. 7). It has been speculated that the mechanism of ClpP activation by ClpX would imply similar conformational changes\(^ {16,49}\).

Our ClpXP1–2 structure demonstrates that this is not the case. ClpP is in the active extended conformation, similar to its conformation in the apo state (Fig. 6a,b). Despite the S98A mutation, the catalytic triad is aligned and in its active conformation (Fig. 6d and Supplementary Fig. 5b). The ClpP1–P2 heptamers are interconnected via typical interactions of antiparallel β9 strands, characteristic for the ‘extended’ active conformation\(^ {43}\) (Fig. 6c). Importantly, the axial pore of ClpP is not widened, compared with the crystal structure of B. subtilis ADEP-bound ClpP (Fig. 6c and Supplementary Video 2). A comparison of the interface between the IGF loop and ADEP with the hydrophobic ClpP pocket reveals that both interact with the same nonpolar residues, including Ile88, Leu49, Tyr63, Phe83, Ile90 and Leu115 (Fig. 7a–c). However, binding of ClpX does not induce the rotation of Tyr63 (Fig. 7c), which is key to opening the pore. Thus, despite the fact that ADEPs and ClpX share the same binding sites, ClpX does not induce the conformational changes resulting in the opening of ClpP. Instead, binding does not induce any major conformational changes, and the diameter of the ClpP channel is sufficient to accommodate the unfolded peptides that are threaded into the ClpP pore by the chaperone to be processed sequentially within the chamber of the peptidase (Fig. 7f).

**Discussion**

ClpXP plays a significant role in the production and regulation of bacterial virulence factors during host infection and is therefore considered a promising target for antimicrobial therapy\(^ {51,52}\). On the other hand, targeting of the mitochondrial homologs is considered a novel approach to halt tumor cell proliferation and metastatic competence\(^ {31,53}\). Despite the important role of ClpXP in protein degradation, biology and medicine in general, structural knowledge of the dynamic two-component proteolytic machinery has lagged behind. The flexible and dynamic interaction between ClpX and ClpP via long flexible IGF and pore-2 loops, involving a symmetry mismatch, together with the asymmetry of the ClpX ATPase make this complex a difficult specimen for structural analysis and probably explain why a high-resolution structure of the complex has been missing so far.

In contrast with previous works, here we utilized the ClpP1–2 heterocomplex from L. monocytogenes, which shows a higher affinity to ClpX than the homocomplex. We mutated the proteolytic site and nucleotide binding site of ClpP1–2 and ClpX, respectively, and cross-linked the sample in order to obtain a ClpXP1–2 complex with superior stability for cryo-EM studies. We believe that this approach was key to determine the ClpXP1–2 structure at an average resolution of 4 Å. The resolution for ClpX, however, is lower and therefore does not allow modeling of side chains.
An interesting finding of the current study is the structural visualization of the interface between the hexameric ClpX ATPase and the heptameric ClpP protease, which involves a symmetry mismatch. The structural plasticity, which is necessary for the interaction of the symmetrically different proteins is provided by the flexibility of the IGF loops. The binding of ClpP to ClpX does not induce major conformational changes to ClpP1−2. Structural superposition of ClpX-bound and unbound LmClpP-S98A (PDB 4RYF) shows low r.m.s. deviation, suggesting that binding of ClpX to LmClpP-S98A does not induce large conformational changes to ClpP1−2. The length of the C terminus is apparently crucial to fine-tune substrate unfolding and translocation. However, further studies are necessary in order to support this scenario.

ClpX is tilted and slightly shifted relative to ClpP2, and the symmetry axes of the protease and the ATPase are therefore not aligned. Thus, upon complex formation, the translocation pathway for unfolded peptides is not straight but twisted. A similar arrangement involving a symmetry mismatch and formation of a twisted peptide translocation channel has been recently described for the PAN proteasome and the bacterial ABC toxin complex. The binding of proteasomal ATPases to the 20S core particle involves a symmetry mismatch and formation of a twisted peptide translocation channel, underlining the dynamic nature of these interactions.

Thus, upon complex formation, the translocation pathway for unfolded peptides is not straight but twisted. A similar arrangement involving a symmetry mismatch and formation of a twisted peptide translocation channel has been recently described for the PAN proteasome and the bacterial ABC toxin complex. The binding of proteasomal ATPases to the 20S core particle involves a symmetry mismatch and formation of a twisted peptide translocation channel, underlining the dynamic nature of these interactions.
Interestingly, the ClpXP1−2 complex from L. monocytogenes dimerizes. Only ClpP2 binds to ClpX, and two opposing ClpX hexamers dimerize head-to-head through the ZBDs. In contrast, the E. coli ClpP homocomplex is doubly capped by ClpX42.1. It is unclear whether the dimerization of the ClpXP1−2 complexes is biologically relevant. The termini of this arrangement of up to four ZBD dimers linking the ClpX hexamers point directly to their distal pore entries. It is therefore tempting to speculate that this interaction might play a role in substrate binding and even help guide it into the ClpX pores. Another explanation might be that, at the high concentrations used for EM, two copies of ClpX might recognize each other as substrate. This scenario is, however, unlikely, because most of ClpX stays intact after incubation of wild-type ClpX with wild-type ClpP1−2.

In summary, the cryo-EM structure of ClpXP1−2 provides the necessary basic insights into ClpXP architecture, essential to understanding the molecular mode of action of this dynamic and highly flexible protein degradation machinery. Our results set the stage for future investigations into conformational changes underlying ClpXP ATP hydrolysis and substrate translocation during protein degradation.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41594-019-0304-0.
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29. Trabuco, L. G., Villa, E., Mitra, K., Frank, J. & Schneider, F. K. and purified proteins, optimized sample preparation, conducted activity assays and gel electrophoresis. S.A.S. and S.R. designed the study. C.G. screened and optimized samples, prepared building ClpX homology models. We are grateful to Dr. M. Haslbeck, G. M. Feind and F. Rührnößl for HDX-MS measurements. This work was supported by the Max Planck Society (to S.A.S.) and the Deutsche Forschungsgemeinschaft (SFB1035) (to S.A.S.).

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Author contributions
S.A.S. and S.R. designed the study. C.G. screened and optimized samples, prepared cryo-EM grids and processed and analyzed cryo-EM data. D.B. cloned, overexpressed and purified proteins, optimized sample preparation, conducted activity assays and gel filtration measurements and analyzed HDX-MS data. C.G. and F. M. built atomic models. C.G. and D.B. prepared figures, C.G., D.B., S.A.S. and S.R. wrote the manuscript. All authors discussed the results.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41594-019-0364-0. Correspondence and requests for materials should be addressed to S.A.S. or S.R. Peer review information Ines Chen was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team. Reprints and permissions information is available at www.nature.com/reprints. Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. © The Author(s), under exclusive licence to Springer Nature America, Inc. 2019
Methods

Cloning. The cloning of pETDuet-1-clpP1−2 and pET300-clpP2−1 were described previously13. ClpP1 and clpP1−2 point mutants, clpP1−2C43−45 and clpP1−2C61−63 were generated using the QuickChange technology. For clpP1−2C43−45 and clpP1−2C61−63 the pETDuet-1-clpP1−2 plasmid was used as a template. ClpP1−2C61−63 and clpP2ΔZBD(E183Q) were obtained with primers containing non-overlapping sequences46. All primers are listed in Supplementary Table 1.

Protein overexpression and purification. ClpP1−2 and its mutants’ variants were overexpressed and purified as follows. The proteins were overexpressed in E. coli BL21(DE3) bearing a pETDuet-1 vector with C-terminally Steg-II-tagged ClpP1 and C-terminally Histagged ClpP2 (ref. 47). The bacteria were grown in LB medium until OD600 0.6 at 37 °C. After induction with 1 mM isopropyl-β-D-thiogalactoside (IPTG), the bacteria were incubated at 37 °C for 6 h. After harvest, the cells were sonicated on ice in lysis buffer (20 mM MOPS, 300 mM KCl, 1% CHAPS, 5% glycerol, pH 7.5; 10 mM imidazole) and then kept at room temperature during the rest of the purification. The proteins from the cleared cell lysate were captured in a HisTrap HP 5 ml column (GE Healthcare) in His buffers (20 mM MOPS, 300 mM KCl, 10% glycerol, pH 7.5; 25 mM HEPES, 200 mM KCl, 1 mM DTT, 0.5 mM ATP, 5 mM MgCl2, 10 mM imidazole, 1% CHAPS, 5% glycerol, pH 7.5), and then kept at room temperature before the rest of the purification. The proteins from the cleared cell lysate were captured in a HisTrap HP 5 ml column (GE Healthcare) in His buffers (20 mM MOPS, 300 mM KCl, 10% glycerol, pH 7.5; 25 mM HEPES, 200 mM KCl, 1 mM DTT, 0.5 mM ATP, 5 mM MgCl2, 10 mM imidazole, 1% CHAPS, 5% glycerol, pH 7.5). In the case of the cysteine-containing mutants, 1 mM TCEP was added to all buffers.

ClpX(E183Q) and ClpX2VE2(F267C) were overexpressed in E. coli BL21(DE3). An expression construct equipped with an N-terminal His-tag and a TEV cleavage site in pET300 vector was used48. The bacteria were grown in LB medium to OD600 0.7 at 30 °C. After induction with 0.5 mM IPTG, the cells were incubated overnight at 25 °C. After harvest, the cells were resuspended in lysis buffer (25 mM HEPES, 200 mM KCl, 1 mM DTT, 0.5 mM ATP, 5 mM MgCl2, 10 mM imidazole, 5% glycerol, pH 7.6) and lysed via ultrasonication. The cleared cell lysate was loaded on a 5 ml HisTrap HP column (GE Healthcare). The column was washed with ClpX wash buffer (25 mM HEPES, 200 mM KCl, 1 mM DTT, 5% glycerol, 40 mM imidazole, pH 7.6). The protein was eluted with ClpX elution buffer (25 mM HEPES, 200 mM KCl, 1 mM DTT, 5% glycerol, 300 mM imidazole, pH 7.6). The protein fractions were pooled, 1 mM EDTA and TEV protease (1.25 mg/ml) were added and the reaction mixture was incubated at 10 °C overnight. Complete TEV cleavage was verified via intact protein MS. The protein solution was loaded on a Superdex200 20/60 column (GE Healthcare) and eluted in ClpX SEC buffer (25 mM HEPES, 200 mM KCl, 1 mM DTT, 0.5 mM ATP, 5 mM MgCl2, 5% glycerol, pH 7.6). ClpX(WT), ClpX(V264C), ClpX(I265C), ClpX(G266C) and ClpX(F267C) were overexpressed and purified similarly with the following modifications: the buffers contained 1 mM TCEP instead of DTT, and the ClpX wash buffer and ClpX elution buffer contained, additionally, 15% glycerol and pH 7.0. In the case of the cysteine-containing mutants, 1 mM TCEP was added to all buffers.

Electron microscopy. The necessary files for the MDFF runs were set up with VMD74, and all necessary files for the MDFF runs were set up with VMD74, and all simulations were performed in NAMD75, using the CHARMM 36 m force field76 using D7 symmetry, whereas the density of ClpX was scaled in order to put an additional weight on this region during the asymmetric refinement. Finally, both densities (ClpX and ClpP) were combined, and the resulting volume was used as a reference for the subsequent refinement iteration. This procedure was performed during the initial rounds in order to obtain global projection parameters. The user function was not applied during the local refinements. This resulted in a density map with an average resolution of 4 Å, where the resolution of the density decreases toward ClpX (Supplementary Fig. 2). The average resolution was calculated between two independently refined ‘half maps’ at the 0.143 FSC criterion. The estimated accuracy of rotation and translation search during the last refinement round was estimated to 1.78 and 1.02 pixels, respectively. Local resolution was computed using the ‘Local Resolution’ tool in SPHIRE. Three-dimensional clustering into four groups was performed using the ROSORT3D tool of SPHIRE. However, according to the ANOVA analysis, the resulting volumes were not reproducible and were therefore not considered for further analysis. Three-dimensional refinement and clustering focusing on the density of ClpP, after removing the ClpP signal from the dataset, also did not result in further improvement of the ClpP density. The density of ClpP was auto-sharpened locally using phenix.auto_sharpen62 and filtered to its average resolution of 3.9 Å. The ClpP density was filtered to an average resolution of 6.5 Å and sharpened with an ad-hoc b-factor of −240 Å. Angular distribution plots were computed using SPHIRE. Sharpened 2D class averages were computed with 3.2 Å members per group.

Atomic modeling. We built a homology model of ClpX with SWISS-MODEL57 using ADP-bound E. coli ClpX (PDB 3HWS, Chain A) and ATP-bound E. coli ClpX (PDB 481B, Chain B). We then used UCSF Chimera63 to fit the structures of ClpX homology model and ClpP1−2 (PDB 1B37, ref. 39) into the cryo-EM density. We used the RosettaES protocol69 to build the missing residues 9–16 for each ClpP2 subunit. Residues 1 and 2 were manually built in Coot52.

With the complete model, we performed several iterative runs of molecular dynamics flexible fitting (MDFF)77 and manual adjustment with Coot, paying particular attention to the fitting of the IFG loops. In the IFG loop, we imposed six-fold symmetry to ClpX, allowing regions poorly supported by the density to settle into reasonable conformations. This restraint was later removed. For the final iterations, we also included steps of manual adjustment in ISOLDE72 and real-space refinement in Phenix78 to decrease the number of Ramachandran outliers and to fit the atomic B factors.

The necessary files for the MDFF runs were set up with VMD74, and all simulations were performed in NAMD75, using the CHARMM 36 m force field76 with the implicit solvation model implemented in NAMD.

For the proper modeling of the structure with MDFF, we included all missing regions of the structure, even if their density did not allow full atomic modeling. As only these regions were removed, we considered that only these regions from the 3D model were in the density. The quality of this model was assessed in Phenix, using the Molprobity77 and EMRinger scores78, as well as the overall geometry of the structure.

Sequence conservation was analyzed with the ConSurfserver79. Analysis of the channel property was performed with ChEvVis80. Electron density maps and models were visualized using Chimera81 and Chimera82.
Peptidase assay. In this assay, the degradation of a fluorogenic tripeptide was measured, for which ClpX was not required. A volume of 99 µl of 1 µM ClpP1–2 was incubated in PZ buffer (25 mM HEPES, 200 mM KCl, 5 mM MgCl2, 1 mM DTT, 10% glycerol, pH 7.6) in black flat-bottom 96-well plates for 1 h at 30 °C. One microliter acetylalanyl-homoarginyl-2-aminooctanoyl-7-amino-4-carbamoylmethylcoumarin (Ac-Ala-hArg-2-Aoc-ACC) substrate (10 mM stock in DMSO) was added, and the fluorescence was measured (380 nm, 430 nm) with an infinite M2000 pro plate reader (Tecan) at 30 °C. Data were recorded in triplicate, and two independent experiments were performed. Peptidase activity was determined by linear regression using Microsoft Excel, and plots were made with GraphPad Prism 6.

Protease assay. Protease assays were carried out in white flat-bottom 96-well plates in a final volume of 60 µl (ClpP1–2, 0.2 µM, ClpX, 0.4 µM and ATP regeneration mix (4 mM ATP, 16 mM creatine phosphate, 20 U/ml creatine kinase), 20 U/ml creatine kinase) were pre-incubated for 15 min at 30 °C in PZ buffer. A total of 0.8 µM eGFP-SsrA substrate was added, and fluorescence was measured (485 nm, 535 nm) at 30 °C. Data were recorded in triplicate, and at least two independent experiments were performed. Protease activity was determined by linear regression using Microsoft Excel, and plots were made with GraphPad Prism 6.

ATPase assay. Ninety microliters 2 µM ClpX in ATPase buffer (100 mM HEPES, 200 mM KCl, 20 mM MgCl2, 1 mM DTT, 1 mM NADH, 2 mM phosphoenolpyruvate, 50 µM lactate dehydrogenase, 50 µM pyruvate kinase, 5% glycerol, pH 7.5) was added to a transparent flat-bottom 96-well plate and incubated for 15 min at 37 °C. The reaction was started by the addition of 10 µM ATP in 100 mM HEPES, pH 7.5. Absorption at 340 nm was measured at 37 °C. Two independent experiments with three replicates each were carried out. ATPase activity was determined by linear regression using Microsoft Excel after subtraction of the background signal (measurement without ClpX), and the plot was made with GraphPad Prism 6.

Hydrogen/deuterium exchange mass-spectrometry. HDX-MS experiments were performed using an ACQUITY UPLC M-class system equipped with automated HDX technology (Waters). HDX kinetics were determined by taking data points at 0, 10, 60, 600, 1,800 and 7,200% at 20 °C. At each data point of the kinetics, 3 µl of a solution of 30 µM free ClpP1–2 and free ClpX were analyzed and compared to the (ClpXp1–2), complex (1 µM). The respective protein solutions were automatically diluted 1:20 into 99.9% D2O-containing buffer (25 mM HEPES, 200 mM KCl, 5 mM MgCl2, 1 mM DTT, 10% glycerol, pH 7.6) and incubated for 15 min at 37 °C. The reaction was started by the addition of 10 µM ATP in 100 mM HEPES, pH 7.5. Absorption at 340 nm was measured at 37 °C. Two independent experiments with three replicates each were carried out. ATPase activity was determined by linear regression using Microsoft Excel after subtraction of the background signal (measurement without ClpX), and the plot was made with GraphPad Prism 6.
Statsitics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☑ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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Software and code

Policy information about availability of computer code

Data collection
i-control 2.0 was used to collect the data for the photometric assays.
EPU software [FEI] was used to collect cryoEM data.
UNICORN 5.11 was used to record the size exclusion chromatograms.
ImageQuant LAS 4000 1.2 was used to scan the SDS-PAGE gels.
Masslynx V 4.1 was used to collect HDX-MS data.

Data analysis
HDX-MS data were analyzed with the PLGS 3.0.3 and DynamX 3.0 software packages and custom MATLAB and python scripts. Data were visualized with OriginPro 2016.
The photometric assays were analyzed using Microsoft Excel 2013 and plots were made with GraphPad Prism 6.
Size exclusion data was analyzed with GraphPad Prism 6.
SDS-PAGE gel image was edited with GIMP 2.8.
Movie frames t were aligned and weighted with Motioncorr 1 and Unblur 1.0.2, respectively.
Single particles of CipoX1/2 dimers and CipoX1/2 were selected with EMAN2’s neuralnet e2boxer and CRYOLO v1.0.0, respectively.
Single particle image processing was performed with SPHERI v1.0 and 1.1
Molecular dynamics simulations were performed with NAMD 2.12 and VMD 1.9.3.
Atomic model refinement was performed with Rosetta 2017.08 and 2016.32.
Molecular dynamics simulations were performed with NAMD 2.12 and VMD 1.9.3.
Atomic model refinement was performed with Rosetta 2017.08 and 2016.32 and the resulting structure was further edited and optimized with Coot 0.87 and PHENIX v1.14.
Atomic coordinates were visualized with Chimera 1.11.2 and molecular figures were created using ChimeraX 1.11.2 and ChimeraX 0.6.
Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

CryoEM structure of LmClpXP1/2 and the molecular models of LmClpX and LmClpP1/2 were deposited at the EMDB and PDB with the accession codes EMDB-10162, PDB-65F5 and PDB-65FX, respectively.

Source data for figure 4, figure 5b-c, Supplementary figure 1a and Supplementary figure 6 are available with the paper online.

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**Sample size**
HDX-MS experiments were performed in duplicate, as is standard in the field.
Photometric assays were performed twice in triplicates, as is standard in the field.

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Photometric assays: All attempts of replication were successful.
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