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Allosteric coupling between α-rings of the 20S proteasome

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Proteasomal machinery performs essential regulated protein degradation in eukaryotes. Classic proteasomes are symmetric, with a regulatory ATPase docked at each end of the cylindrical 20S. Asymmetric complexes are also present in cells, either with a single ATPase or with an ATPase and non-ATPase at two opposite ends. The mechanism that populates these different proteasomal complexes is unknown. Using archaea homologs, we construct asymmetric forms of proteasomes. We demonstrate that the gate conformation of the two opposite ends of 20S are coupled: binding one ATPase opens a gate locally, and also opens the opposite gate allosterically. Such allosteric coupling leads to cooperative binding of proteasomal ATPases to 20S and promotes formation of proteasomes symmetrically configured with two identical ATPases. It may also promote formation of asymmetric complexes with an ATPase and a non-ATPase at opposite ends. We propose that in eukaryotes a similar mechanism regulates the composition of the proteasomal population.
In eukaryotic cells, 26S proteasomes are complex protein machines that execute regulated and ATP-dependent intracellular protein degradation. Its functions include degrading and controlling the amounts of regulatory proteins, such as those involved in cell cycle regulation. The 26S proteasome is composed of the 20S core particle (CP), symmetrically capped at each end by a 19S regulatory particle (RP). The proteolytic module, the 20S CP, is a hollow barrel-shaped chamber formed by four stacked heptameric rings, two inner β-rings and two outer α-rings. The proteolytic sites at N-termini of the β-subunits are sequestered within the chamber. Unfolded substrates enter the chamber through a narrow pore in the center of α-rings, where N-termini of α-subunits form a gate to impede unregulated access to the proteolytic chamber. The primary role of 19S RP is to recognize, unfold, and translocate targeted substrates into CP for degradation. In addition, the six proteasomal ATPases (Rpt subunits) within 19S RP engage with the α-ring of 20S CP and open its gate for unfolded substrates to enter the proteolytic chamber and, presumably, for small peptide products of proteolysis to be released from the chamber.

The canonical 26S proteasome has a 2-fold symmetry, with a 19S cap at each end of the 20S. CPs capped by a single 19S, or uncapped, are also abundant in cells. Other types of non-ATPase RP complexes are also present in eukaryotes, such as PA28 and PA200. Thus, there are hybrid particles, in which 20S is capped at each end by a different RP. The functions of these non-ATPase activators in the regulated protein degradation process remain elusive, but invariably include opening the α-ring gate. Consequently, as defined by alternative cap configurations, proteasomes constitute a diverse family. A key question is in what way these structural alternatives differ in their biochemical capacities and cellular functions. More specifically, how do 20S symmetrically capped with two 19S compare with those bearing a single 19S? Is one cap sufficient to make the proteasomal particle fully functional? Furthermore, are the effects of RP docking on the α-ring purely local, or do such effects propagate distantly? Such questions are not readily addressed with eukaryotic 26S proteasomes, because it is difficult to biochemically separate canonical symmetric 26S particles from those with only one 19S for functional studies.

In this study, we use archaeal proteasomes to examine these long-standing questions. Specifically, we ask whether substrate processing efficiency is enhanced by the presence of two RPs and whether gate opening can be rate determining. As a model system, the archaeal proteasome is similar to but simpler than eukaryotic proteasomes. In archaea, the 20S is composed of homo-heptameric α- and β-rings, and PAN (Proteasomal Activator Nucleotideide), a homohexamer proteasomal ATPase, is the RP found in many species. Archaeal PAN ATPase, like the ATPase ring of eukaryotic 19S, docks to the α-ring via a C-terminal HbYX (hydrophobic, tyrosine, any amino acid) motif. In the past, many mechanistic insights relevant to eukaryotic 26S proteasome were derived from studies of archaeal proteasomes.

We designed and assembled in vitro various asymmetric archaeal 20S, in which one of the two α-rings contains a point mutation, K66A, which prevents RP binding to the α-ring. Thus, only one α-ring in such an asymmetric 20S can bind to a specific RP. This approach enabled us to measure and compare proteolytic activities of singly capped 20S versus doubly capped ones, and to reveal structurally evident allosteric coupling between the two separate α-rings upon RP binding. Our studies reveal that, upon binding of a RP with a conserved C-terminal HbYX motif to one α-ring, the gate of the opposite α-ring opens allosterically, resulting in similar proteolytic activities of 20S singly capped and doubly capped with PAN. Such allosteric regulation of the α-ring gate requires the C-terminal HbYX motif to be present in RP. Furthermore, allosteric coupling between two α-rings leads to cooperative binding of activator to 20S. We hypothesize that there is a similar allosteric coupling between the two α-rings in eukaryotic 26S proteasomes.

**Results**

**In vitro assembling of asymmetrical archaeal 20S proteasomes.** Functional archaeal 20S CPs can be assembled in vitro by mixing and incubating purified full-length α-subunits and propeptide truncated β-subunits that are individually expressed in *E. coli* (Supplementary Fig. 1). When expressed and purified alone, α-subunits assemble into homo-heptameric rings. Two α-rings come together back-to-back, forming a tetradecameric double α-ring, with the α-subunits in each ring interdigitated with other α-subunits, similar to the manner in which they interdigitate with β-subunits in an intact native CP (Supplementary Fig. 1c-i and Supplementary Table 1). Mixing such double α-rings with β-subunits, which remain as monomers throughout the purification, induces separation of the α-tetradecamer double rings into two heptameric α-rings and results in the assembly of α- and β-subunits together into intact CPs. Both negative stain and single-particle cryo-electron microscopy (cryo-EM) show in vitro assembled CPs are indistinguishable from the native recombinant CP assembled in vivo (Supplementary Figs. 1j and 2). We also confirmed that the in vitro assembled CPs are proteolytically active and its α-ring gate can be activated by proteasomal activators, demonstrating that our in vitro assembled CP is functional and active (Supplementary Fig. 1k).

Because α-rings can be pre-assembled homogeneously with the same type of α-subunit, it is possible to mix two different types of pre-assembled α-rings with β-subunits to assemble CP with two different types of α-ring at its opposite ends. We generated two different types of α-rings with different affinity tags: one was made by co-expressing two full-length α-subunits in one expression plasmid, one with a His-tag and the other with an MBP tag, both at the C-terminus. Such assembled α-rings thus contain a mixture of two different affinity tags to facilitate multistep affinity purification. A second type of α-ring was made with a single Strep-Tactin tag. (We found that Strep-Tactin tags can only be used once.) The α-ring gate was placed before all α-subunits in each ring interdigitated with other α-subunits, similar to the manner in which they interdigitate with β-subunits in an intact native CP (Supplementary Fig. 1c-i and Supplementary Table 1). Mixing such double α-rings with β-subunits, which remain as monomers throughout the purification, induces separation of the α-tetradecamer double rings into two heptameric α-rings and results in the assembly of α- and β-subunits together into intact CPs. Both negative stain and single-particle cryo-electron microscopy (cryo-EM) show in vitro assembled CPs are indistinguishable from the native recombinant CP assembled in vivo (Supplementary Figs. 1j and 2). We also confirmed that the in vitro assembled CPs are proteolytically active and its α-ring gate can be activated by proteasomal activators, demonstrating that our in vitro assembled CP is functional and active (Supplementary Fig. 1k).

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Using this strategy, we assembled 20S that contain two distinct α-rings at their opposite ends. One end is wild type and the other contains the point mutation K66A. Lys66 residues in the α-ring form a salt bridge with C-terminal carboxylate of activators, PA26 or PAN, and a point mutation of this residue to Alanine or Serine abolishes binding of activators. Thus asymmetric CP, named αWTβ4K66A, allow the binding of an activator to the wild-type α-ring but not to the opposite α-ring with the K66A mutation, which impairs binding of any activators, including both proteasomal ATPases with a C-terminal HbYX motif or 11S
activators such as PA26 from *Trypanosoma brucei*18,20. This allowed formation of a homogeneous population of singly capped proteasomes. As a control, we also assembled in vitro symmetric wild-type CP αααβββααα for comparison. A full listing of in vitro assembled complexes is shown in Fig. 1b.

In this study, we used three types of activators. The first one is a wild-type homo-hexameric PAN ATPase, homologous to the Rpt ATPase subunits in the base of 19S in eukaryotic 26S proteasomes. The C-terminus of PAN contains an HbYX (hydrophobic, tyrosine, and any amino acid) motif that is required for binding to the α-ring and opening its gate18. When PAN is used, we also introduced a point mutation R28L to the corresponding α-ring of 20S CP. This mutation does not influence the gating or proteolytic activity but enhances binding of PAN to CPs. The second activator is heptameric PA26 with the point mutation E102A abolishing the capability of PA26 to activate 20S proteasomes. As a control, we also assembled in vitro symmetric αααβββααα, which we named PA26PANc. PA26 is a heptameric 11S activator from *Trypanosoma brucei*23. The point mutation E102A abolishes a specific interaction between the so-called activation loop of PA26 with a reverse turn loop in the 20S α-subunit, thus abolishing the capability of PA26 to activate 20S proteasomes by opening its gate. Instead, the HbYX motif from the PAN C-terminus placed at the heptameric PA26PANc C-termini performs gate opening12. As demonstrated previously, PA26PANc activates the CP particle in the same manner as PAN, except that it symmetrically engages the 7-fold symmetric α-ring, and thus avoids a symmetry mismatch between the α-ring and the activator19,22. The third activator is PA26 with a single point mutation V230F, which functions like the wild-type PA26, but with an enhanced binding affinity to the archaeal 20S CP22 (Supplementary Table 2). Mixing our in vitro assembled CP with these activators generated the desired types of single or double capped proteasomes (Fig. 1c and Supplementary Fig. 3).

**Proteolytic activities of asymmetric 20S proteasome.** We aimed to use the in vitro assembled asymmetric CP to address the question of whether the singly capped proteasome has the full peptidase or protease capacity of a doubly capped one. To this end, we used two types of substrate, LFP and GFP-ssrA. LFP is a fluorogenic peptide of nine amino acids that emits florescence upon proteolytic cleavage. It can diffuse into the archaeal 20S and undergo proteolysis even without an activator, but gate opening increases its entry rate. Upon opening the α-ring gate, fluorescent product is seen to accumulate at a faster rate, consistent with an increased flux of LFP peptides into the 20S degradation chamber18. The relationship between the peptidase activity and the amount of activator allows an estimation of the binding affinity of the activator to CP (Supplementary Fig. 4 and Supplementary Table 4). GFP-ssrA is a folded protein substrate containing the very stable and bulky GFPβ-barrel, which cannot passively diffuse into the 20S for degradation, even when its gate is open. Degradation of GFP-ssrA requires a CP in complex with PAN ATPase; PAN hydrolyzes ATP to unfold GFP-ssrA and actively translocate the unfolding polypeptide into the contiguous CP, where proteolysis takes place.

Using PA26PANc as the activator, we first compared the peptidase activities of the asymmetric CP with that of the symmetric CP. We incubated separately 16 nM asymmetric and symmetric 20S CP with 112 nM PA26PANc, so that a high percent of 20S CP are either singly capped as PA26PANc-αααβββααα or doubly capped as PA26PANc-αααβββααα. Asymmetric PA26PANc, as confirmed by negative stain EM (Supplementary...
were prestained before applying anti-GFP antibody. The experiment was repeated two times; each produced similar results. Supplementary Fig. 5). We found that PAN- and western blot analysis against GFP (Fig. 2b, c and Table 1).

Table 1 Summarization of the $k_{cat}$ (1/min), $K_M$ (μM), and $V_{max}$ (μM/min) of LFP peptide degradation by symmetric and asymmetric 20S CP without and with activators.

| 20S CP       | $k_{cat}$ (1/min) | $K_M$ (μM) | $V_{max}$ (μM/min) |
|--------------|------------------|------------|-------------------|
| WT-PANc      | 4.13 ± 0.37      | 5.80 ± 1.53| 66.29 ± 5.59      |
| K66A         | 12.44 ± 1.19     | 11.17 ± 2.39| 199.1 ± 19.1      |
| WT-PANc      | 14.40 ± 1.10     | 5.96 ± 0.96 | 82.28 ± 4.51      |
| K66A         | 19.27 ± 2.65     | 19.27 ± 2.65| 230.5 ± 17.5      |

Fig. 2 Substrate degradations by asymmetric proteasomes. a) Kinetics of LFP peptide degradation by symmetric (blue) and asymmetric (yellow) 20S CP alone and with double (black) and single (red) activator PA26PANc. Three (n = 3) biologically independent experiments were carried out. The means are used to derive the curve and the error bars represent standard deviation. b) Time course of GFP-ssrA degradation by PAN–αWTββα66A and PAN–αWTββα66A, analyzed by western blotting with anti-GFP antibody. The MW reference proteins were prestained before applying anti-GFP antibody. The experiment was repeated two times; each produced similar results.

so, the overall rate of peptide hydrolysis would not be greatly influenced by whether one versus two gates are open. An alternative explanation is that binding of an activator to the proximal α-ring is sufficient to allosterically open the gate of the opposite α-ring (named distal α-ring or gate). In this scenario, binding a single RP opens two gates. This idea was tested by determining the gate conformation of distal α-rings in asymmetric proteasome complexes.

Distal gate conformation in PA26PANc-associated proteasomes. We determined a number of single-particle cryo-EM structures of the in vitro assembled asymmetric CP with one activator bound, PA26PANc–αWTββα66A and PAN–αWTββα66A (Supplementary Table 3). We did so in the presence or absence of substrates to test whether substrate processing would affect the outcome. When determining these reconstructions, we applied a C7 symmetry. The proximal and distal α-rings were identified easily using the bound activator as a reference. The resolution of all reconstructions was around 3 Å, sufficient to reveal the gate conformations of each of the two α-rings, proximal and distal.

In eukaryotic 20S proteasomes, the α-ring contains seven homologous but different α-subunits, each with a different N-terminus. Through a complex hydrogen bonding network, these N-termini form a very ordered gate4. In archaea 20S proteasome with homo-heptameric α- and β-rings, N-termini of the seven identical α-subunits also form a gate that prevents bulky substrates, such as long peptides or folded proteins, from entering the 20S proteasome for degradation18. The N-termini of the seven α-subunits cannot form a structurally well-ordered gate through hydrogen bonds. In all structures of archaeal 20S proteasomes with a closed gate, the first nine residues of α-subunits are typically not resolved19,20. NMR spectroscopy analysis of gate conformations of archaeal 20S shows that N-terminal tails of α-subunit proteins point downwards in a closed conformation and upwards in an open conformation. However, because of spatial constrains, not all N-termini of an α-ring in a closed gate can be accommodated in a downward conformations21. In our structural studies, we used the same criterion, tracing the pointing direction of the N-terminal tails of...
the α-subunits to evaluate the gate conformation. This is based on previous single-particle cryo-EM studies of gate opening in archaeal 20S and NMR studies of archaeal 20S with open and closed gates (Supplementary Fig. 6a, e). Here, we used the same criterion to assign the gate conformation of the distal α-ring when the proximal α-ring is bound with an activator (Supplementary Fig. 6).

We first determined the structure of PA26PANc-αWTββαK66A to resolutions of 2.9 Å without LFP substrate and 3.4 Å with LFP (Fig. 3 and Supplementary Fig. 7). In both structures, the conformation of the proximal α-ring and two β-rings match very well with the crystal structure of wild-type symmetric 20S proteasome in complex with the same activator (PDB: 3IPM). As shown in this crystal structure, binding the hybrid activator PA26PANc to the α-ring causes α-subunit rotation, which opens the gate (Fig. 3c–e). In both our structures (with and without LFP), the distal α-ring, without an activator, also shows features that match an open gate, with all N-termini moved away from the pore and pointing upwards (Fig. 3f, h). However, unlike the proximal gate, there is no rotation of distal α-subunits (Fig. 3g). Rather, the conformation of this α-ring matches well with that in the crystal structure of archaeal 20S proteasome in complex with PA26 (PDB: 1YAR20), except that the gate is in an open conformation. The surprising observation that binding of PA26PANc to the proximal α-ring opens the gate of the distal α-ring is consistent with allosteric action. That the opening of the distal gate is independent of the presence of substrate implies that substrate presence or its active proteinolysis within the β-ring does not mediate the observed allosteric effect.

Distal gate conformation in PAN-associated proteasomes. We also determined structures of PAN-αWTββαK66A in the presence of ATP, without and with substrate GFP-ssrA, both to resolutions of 3.4 Å (Fig. 4 and Supplementary Fig. 8). Because of the symmetry mismatch between the heptameric α-ring and hexameric PAN ATPase, PAN does not engage with α-ring symmetrically, and not every binding pocket in the CP is occupied by a C-terminus of PAN. Furthermore, engagement of PAN and α-ring is heterogeneous among different particles17 and such heterogeneity limits the achievable resolution. To obtain sufficient resolution to evaluate the gate conformation, we applied a C7 symmetry. Thus, the density of PAN in this reconstruction does not represent its correct structure. Since it is not the emphasis of this study, we did not attempt to obtain a high-resolution structure of PAN bound with the CP. The conformation of proximal gate (and presumably the distal gate as well) is an average of slightly different conformations of α-subunits within the ring. Even so, the conformation of the gate in the C7 symmetrized density map still represents the state of the gate. Whether without or with the substrate, the proximal gates are in an open conformation, consistent with the gate opening by the C-terminus of PAN with its native C-terminal HbYX motif. Furthermore, the C7 symmetry averaged density map still shows rotation of α-subunits induced by binding of PAN’s C-termini. This result (Fig. 4) using PAN is consistent with the structures determined with PA26PANc (Fig. 3). Thus, structures of PA26PANc-αWTββαK66A and PAN-αWTββαK66A both show that the gate of the distal α-ring is open even without direct association with an activator; this distal gate is opened allosterically by the engagement of an activator to the proximal α-ring. These data indicate that an RP carrying the HbYX motif, which intercalates within the pockets between neighboring pairs of α-units, and induces a small rotation in the α-unit to open the gate, can mediate allosteric gate opening.

Distal gate in proteasomes using PA26 without HbYX motif. To test whether the HbYX motif is a required element for inducing allosteric communication between the two distant α-rings, we further determined the structure of the asymmetric CP αWTββαK66A in complex with a single PA26, which does not have an HbYX motif grafted to its C-terminus. As expected, the gate in the proximal α-ring is open and there is no rotation of the α-subunit, but the activation loop of PA26 is engaged with the
reverse turn loop of CP (Fig. 5 and Supplementary Fig. 9). In contrast to the results using PAN and PA26PANc, in an asymmetric proteasome bearing PA26, the gate of the distal α-ring is closed. This result suggests that binding a proteasomal activator without a C-terminal HbYX motif to one α-ring opens the proximal gate only, but does not induce allosteric opening of the distal gate.

These results motivated us to further test the peptidase activities of asymmetric CP with a single capped PA26, in comparison with that of the symmetric CP with two PA26 caps. If the diffusion rate of LFP peptide through the open gate into CP is a rate-limiting step, the peptidase activities of symmetric CP double capped with PA26 (two gates open) should be higher than that of the asymmetric CP capped with only one PA26 (one gate open). Indeed, the peptidase activity of symmetric CP doubly capped by PA26 is about twice that of the singly capped asymmetric CP (Fig. 5g and Table 2), as predicted from the structures and the stated hypothesis. This result further suggests that the rate of unfolded peptide substrates diffusing through an open gate into the proteolytic chamber is a rate limiting step for the peptidase activity of the 20S proteasome.

Cooperative binding associated with distal gate opening. Binding of a regulatory cap to an α ring promotes local gate opening. In case of activator with a C-terminal HbYX motif, such as PAN or PA26PANc, the distal gate is also opened allosterically. Such an unoccupied α ring with an allosterically opened gate, in which N-termini of all α subunits are moved away from the pore and pointing upwards, may kinetically favor activator binding over an α ring that is otherwise identical but with its gate closed. The empty and open distal gate is preconfigured to accept an activator. This hypothesis makes a testable prediction: PAN or PA26PANc binding to CP is cooperative, but PA26 binding to CP is not cooperative. Cooperative binding of an RP implies that the distribution among uncapped, singly capped and doubly capped particles will show a deficit of singly capped CP, and a corresponding increase in uncapped and doubly capped particles. Interestingly, such cooperative binding of eukaryotic 19S to 20S has been described for the eukaryotic proteasome.

To test this hypothesis, we collected electron micrographs of negatively stained wild-type symmetric CP incubated with either PA26PANc or PA26 in two different molar ratios, 4:1 and 3:1. We picked all CP particles, with or without caps, and classified them into three categories: CP alone and with one or two activators bound (Supplementary Fig. 10). The probability of a cap binding to an α ring, P, was estimated from the number of particles in each category. The hypothesis of non-cooperative binding of cap to CP predicts a binomial distribution of particles in each category: \( P^2 \) for doubly bound CP, \( 2P(1-P) \) for singly bound CP, and \( (1-P)^2 \) for unbound CP (Table 3 and Supplementary Tables 5 and 6). Comparing the observed and the predicted number of singly capped particles, singly capped PA26PANc is significantly less than that predicted by the null hypothesis. Such a deficit of singly capped complexes is the hallmark of positive cooperativity. The null hypothesis was rejected with high probability by \( \chi^2 \) analysis. However, a similar analysis with PA26 revealed no evidence of positive cooperativity. The number of observed particles among the three complexes is quite close to a binomial distribution, supporting the null hypothesis of no cooperativity. Overall, these results support the conclusion that a preformed unoccupied open gate in the α ring has increased affinity for binding RP. Cooperative RP binding and distal gate opening are thus seen to be different aspects of a single underlying mechanism.

Discussion

In both eukaryotes and archaea, 20S is 2-fold symmetric, with identical α-rings at each end; these can bind to identical or different proteasomal activators. In the case of the most widely studied eukaryotic 26S proteasome, two 19S activators are symmetrically attached to the CP. However, a significant fraction of 20S of eukaryotic cells are capped with only one 19S, forming asymmetric proteasomal complexes. In addition, there are also 20S capped asymmetrically by a 19S ATPase activator and a
non-ATPase activator, such as 11S PA28-hybrid proteasomes. It has been unclear whether proteasomes function similarly with one or two caps. This has been difficult to test biochemically, because homogeneous native proteasomal complexes with only one activator cannot be readily purified. Our in vitro assembled asymmetric CP enabled us to generate homogeneous singly capped proteasomal particles. The biochemical data presented here show that, when bound with activator that has an HbYX motif at its C-terminus, single and double capped CP have similar substrate degradation rates, both for small peptide substrates and protein substrates that require ATPase-dependent unfolding. However, when bound with PA26, which does not have a C-terminal HbYX motif, peptidase activity of doubly capped proteasomes is greater than for those with a single cap. Consistently, our structural and cooperativity binding analyses revealed that allosteric opening of the distal gate by engagement of an activator to the proximal α-ring and cooperative binding of an activator to the CP are observed when an HbYX motif is present in the C-terminus of the activator.

In archaea, only proteasomal ATPases (PAN or Cdc48) are known to contain such a native HbYX motif at its C-terminus. As shown here, that motif functions similarly when artificially grafted to the C-termini of the non-ATPase activator PA26. Previous studies showed that binding of an activator with a C-terminal HbYX motif causes a rotation of the α-ring. The capacity of the HbYX motif to allosterically open the distal gate suggests that the rotation may play a role in initiating the allosteric action. The present observation, that engaging an activator to one α-ring is propagated to the opposite α-ring, is remarkable, as it implies action at a great distance, across the full span of the CP, some 150 Å. The allostery must necessarily be transmitted by a series of conformational changes within the β-rings separating

### Table 2

Table summarizes the kinetic parameters of peptidase activity of symmetric and asymmetric 20S CP without and with PA26 activator.

| 20S CP activator | α<sub>wt</sub>ββα<sub>wt</sub> | α<sub>wt</sub>ββα<sub>K66A</sub> | α<sub>wt</sub>ββα<sub>wt</sub>PA26 | α<sub>wt</sub>ββα<sub>K66A</sub>PA26 |
|------------------|-----------------|-----------------|-----------------|-----------------|
| α<sub>cat</sub> (1/min) | 7.89 ± 0.38 | 50.34 ± 4.91 | 5.82 ± 0.46 | 25.54 ± 2.61 |
| K<sub>M</sub> (μM) | 4.14 ± 0.65 | 37.31 ± 5.24 | 7.06 ± 1.41 | 24.48 ± 4.05 |
| V<sub>max</sub> (μM/min) | 126.2 ± 6.1 | 805.4 ± 78.6 | 93.2 ± 7.3 | 408.6 ± 41.7 |

![Diagram of proteasome complexes](image)
the non-contiguous α rings in the CP. However, except for the changes of N-terminal gating residues of α-subunits, we failed to detect any noticeable conformational change in the β-rings sandwiched between the two α-rings. Therefore, how the β-rings transmit the conformational changes from the proximal α-ring to the distal one remains to be determined.

Previous descriptions of allosteric interactions within the euarkaryotic proteasome CP predominantly focused on how the conformational changes associated with substrate engagement with β ring is propagated to the α ring and alters its interaction with RP27–31. The findings described here extend that allosteric coupling across the full expanse of the CP, from one α ring to the other at the opposite end. Distal gate opening is observed regardless of whether substrate is present and being processed.

Allosteric communication between two oppositely positioned α rings is reflected by two sorts of observation: cooperative binding and distal gate opening by an ATPase regulatory particle with an HbYX motif. These forms of regulation can be understood mechanistically as one: a preformed open gate in an α-ring confers a higher affinity to an ATPase RP than does an α-ring with a closed gate, thus promoting the formation of a proteasomal complex with doubly capped ATPase. The two findings – cooperativity and distal gate opening – may thus be regarded as real manifestations of a common underlying mechanism.

We propose that the mechanism explanatory of our observations of PA26PANc cooperative binding suggests that a non-ATPase RP, one without a C-terminal HbYX motif, will also have a higher affinity to an α-ring with a preformed open gate. If so, mixing 20S with a combination of ATPase and non-ATPase activators would bias the population of proteasomal complexes towards either those doubly capped with ATPases, or hybrid complexes with an ATPase and a non-ATPase at the two opposite ends. Within euarkaryotic cells, such cooperation might promote, for example, the formation of either 26S doubly capped with two 19S or hybrid proteasomes capped with a 19S and a non-ATPase activator, such as 11S or PA200, and at the same time minimize formation of proteasomes doubly capped with two non-ATPase activators. Such proteasomal particles doubly capped with non-ATPase activators have no obvious functionality in degrading protein substrates.

From the prospective stated, RPs may be regarded as falling into two classes – those that can act as both transmitters and recipients of allosteric information (as here documented for RPs with an HbYX motif) and those that are solely recipients (RPs that lack the motif). Proteasomes are modular molecular machines. The presence within an individual cell of multiple alternate RPs, some functioning dually as transmitters and recipients of allostery, others solely as recipients, enhances the potential number of stable CP-resident molecular species, thus regulating how CP pools partition among these alternate populations. Concentrations of the individual RP modules and their absolute and relative affinities for the CP α ring must be important determinants of how a CP population is partitioned among alternate forms. Cooperative binding provides an additional form of regulatory complexity.

We have examined cooperativity in a highly constrained experimentally accessible system, looking at interactions among pure populations of archaea CP in the presence of a single species of RP, here specifically PA26PANc or PA26. In this simple context, cooperativity has a simple consequence: a deficit of singly capped species and concomitant excess of uncapped and doubly capped. Our assays of various biochemical activities have revealed that adding a second identical cap provides little enhancement of activity, at best 2-fold. Such a small change in biochemical outcome seems an implausible foundation to support a conserved complex allosteric interaction, one common to both eukaryotes and archaea. Cooperativity more plausibly promotes formation of proteasome species with hybrid capping, in which distinct RP complexes inhabit the same proteasome complex, for both archaea and eukaryotes. Hybrid asymmetric proteasomes are abundant in cells, but have unclear function. The mechanism of their formation and maintenance is unexplored. Allosteric α ring coupling may provide a means for promoting or fine tuning hybrid formation.

Certain archaea species lack an obvious homolog of PAN. It has been conjectured that they utilize an alternate ATPase activator, homologous to the Cdc48/p97 ATPase of eukaryotes26. Cdc48/p97 contains the C-terminal HbYX motif. It would be of interest to determine whether that activator can also confer distal allosteric gate opening.

Our studies were carried out using archaea 20S proteasome, because it is feasible to assemble asymmetric 20S CP in vitro. Part of our findings, that non-ATPase activator does not activate 20S CP allosterically, is consistent with other prokaryotic proteasome-activator systems, such as in Mycobacterium tuberculosis32,33. Nevertheless, our findings could also shed light on the euarkyotic proteasomal complex, as previous biochemical and structural studies demonstrate similarities between the two. Certainly, euarkyotic proteasomes are more complex. Only three of six Rpt subunits contain the HbYX motif, and full gate opening of the proximal α-ring depends on engagement of substrates34. At present, current single-particle cryo-EM structural studies of asymmetric euarkyotic proteasomes have not determined whether the distal gate or distal α-ring is allosterically influenced by the functional state of the proximal activator, as the full array of experimental conditions relevant to this question have not been explored. Further investigation would be required to fully address the questions posed here.

### Methods

#### Protein expression and purification.
Recombinant wild-type and mutant forms of Thermoplasma acidophilum 20S core particles (CP), its individual α- and β-
subunits, and various proteasomal activators (PAN ATPase from *Methanococcus jannaschii*, and PA26 from *Trypanosoma brucei*) with various affinity tags were expressed in E. coli BL21(DE3) using the plasmid pET20S, pET20S_Mb and pET20S_Hapia (Supplementary Table 1). A TEV proteolytic site was inserted adjacent to tags for tag removal after final purification and complex assembly. After culture in LB broth at 37 °C, expression was induced with 0.5 mM IPTG at a cell density of OD 0.6–1.0, and cells further cultured at 18 °C overnight before harvest. Cells were lysed by emulsifier ultrasonicator and sonication and affinity purification performed using methods appropriate to the specific tags. Purification of individual proteins was assessed by SDS–PAGE and Coomassie staining. Fully functional symmetric 20S CP was assembled by mixing individually expressed α- and β-subunits. Residues 2–8 of the β-subunit were truncated to simulate the autoproteolytic N-terminal pro-peptide truncation that occurs during native CP assembly.

**In vitro reconstitution of asymmetric 20S CP**. In vitro assembly and purification of 20S CP with the intended compositions were performed using a multi-step affinity purification. Specifically, two different types of individually purified α- and β-subunits were incubated with β-subunit in a 1:α (type 1:2β);[β (type 2) molar ratio] in Tris buffer at pH 7.5 for 1 h at 37 °C. Typically, the mixture contains different unstructured single or double α-rings (α2, α3, α4, α5, and α6), asymmetric 20S CP and free β monomers. Affinity purification with strep-trap column select all assembled or individual α-subunit containing α6. After strep-trap column, a TEV protease enzyme was used to cleave all the tags except MBP, followed by another affinity purification by MBP column. In the final assembly step, a symmetric 20S CP was collected on a C4 column, which yields fully assembled asymmetric 20S CP. The functionality of 20S CP with the intended compositions were performed using a multi-step affinity purification by MBP column in stoichiometric excess (10:1, RP: CP).

To assemble complexes of asymmetric 20S CP with PA26PANc or PA26V230F, regulatory peptides were added to partially purified pre-assembled asymmetric 20S CP before affinity purification by MBP column in stoichiometric excess (10:1, RP: CP). An MBP column was used to capture fully assembled asymmetric 20S CP in complex with RP. To assemble the complex of 20S CP with PAN, in vitro assembled 20S CP, either symmetric or asymmetric, was mixed with PAN in stoichiometric excess (4:1, PAN:CP) in the presence of nucleotide. For ATP-dependent GFP-ssrA degradation assay, ATP was used. For structural study of the 20S-PAN complex, MBP column was not used. Finally, complex products were challenged with negative stain EM to assess whether they had the anticipated structure. Point mutations used in this study are summarized in Supplementary Table 2.

**Peptidase activity assay**. Peptidase activity (Figs. 2a and 3g) was determined by measuring the fluorescent intensity generated by LFP hydrolysis, a fluorogenic 9-residue peptide, as described previously. Assays were performed in 50 μL buffer (25 mM Tris pH 7.5, 10 mM MgCl2, and 5% glycerol) in a volume of 60 μL. Reactions were initiated by adding of GFP-ssrA to a 1.5 μM final concentration of ssrA peptide. The fluorescence intensity was measured at 480 nm excitation and 517 nm emission wavelengths. Each experiment was carried out in triplicate. The data that support this study is available from the corresponding author upon reasonable request. The Cryo-EM maps have been deposited in the Electron Microscopy Data Bank under 20877, 20878, 20879, 20880, and 20881. The atomic models have been deposited in the Protein Data Bank under 6UTF, 6UTG, 6UTH, 6UTL, and 6UTJ. Source data are provided with this paper.
References

1. Voges, D., Zwickl, P. & Baumeister, W. The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. 68, 1015–1068 (1999).

2. Groll, M. et al. Structure of 20S proteasome from yeast at 2.4 A resolution. Nature 386, 463–471 (1997).

3. Lowe, J. et al. Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. Science 268, 533–539 (1995).

4. Groll, M. et al. A gated channel into the proteasome core particle. Nat. Struct. Biol. 7, 1062–1067 (2000).

5. Coux, O., Tanaka, K. & Goldberg, A. L. Structure and functions of the 20S and 26S proteasomes. Annu. Rev. Biochem. 65, 801–847 (1996).

6. Kohler, A. et al. The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate entry and product release. Mol. Cell 7, 1143–1152 (2001).

7. Qian, M. X. et al. Acetylation-mediated proteasomal degradation of core histones during DNA repair and spermatogenesis. Cell 153, 1012–1024 (2013).

8. Dubiel, W., Pratt, G., Ferrell, K. & Rechsteiner, M. Purification of an 11 S regulator of the multicatalytic protease. J. Biol. Chem. 267, 22369–22377 (1992).

9. Ma, C. P., Slaughter, C. A. & DeMartino, G. N. Identification, purification, and characterization of a protein activator (PA28) of the 20 S proteasome (macropain). J. Biol. Chem. 276, 10515–10523 (1992).

10. Cascio, F., Call, M., Petre, B. M., Walz, T. & Goldberg, A. L. Properties of the hybrid form of the 26S proteasome containing both 19S and PA28 complexes. EMBO J. 21, 2636–2645 (2002).

11. Hendel, K. B., Khan, S. & Tanaka, K. Simultaneous binding of PA28 and PA700 activators to 20 S proteasomes. Biochem. J. 332, 749–754 (1998).

12. Kepp, F., Dahlmann, B. & Kuehn, L. Reconstitution of hybrid proteasomes from purified PA700-20 S complexes and PA28alphabeta activator: ultrastructure and peptidase activities. J. Mol. Biol. 313, 465–471 (2001).

13. Rechsteiner, M. & Hill, C. P. Mobilizing the proteolytic machine: cell biological roles of proteasome activators and inhibitors. Trends Cell Biol. 15, 27–33 (2005).

14. Tanahashi, N. et al. Hybrid proteasomes. Induction by interferon-gamma and contribution to ATP-dependent proteolysis. J. Biol. Chem. 275, 14336–14345 (2000).

15. Maupin-Furlow, J. Proteasomes and protein conjugation across domains of life. Nat. Rev. Microbiol. 10, 100–111 (2011).

16. Majumder, P. et al. Cryo-EM structures of the archaeal PAN-proteasome reveal an around-the-ring ATPase cycle. Proc. Natl Acad. Sci. USA 116, 534–539 (2019).

17. Smith, D. M. et al. ATP binding to PAN or the 26S ATPases causes association with the 20 S proteasome, gate opening, and translocation of unfolded proteins. Mol. Cell 20, 687–698 (2005).

18. Smith, D. M. et al. Docking of the proteasomal ATPases’ carboxyl termini in the 20 S proteasome’s alpha ring opens the gate for substrate entry. Mol. Cell 27, 731–744 (2007).

19. Rabl, J. et al. Mechanism of gate opening in the 20 S proteasome by the proteasomal ATPases. Mol. Cell 30, 360–368 (2008).

20. Forster, A., Masters, E. L., Whitby, F. G., Robinson, H. & Hill, C. P. The 1.9 A structure of a proteasome-11S activator complex and implications for proteasome-PAN/PA700 interactions. Mol. Cell 18, 589–599 (2005).

21. Zwickl, P., Kleine, J. & Baumeister, W. Critical elements in proteasome assembly. Nat. Struct. Biol. 1, 765–770 (1994).

22. Yu, Y. et al. Interactions of PAN’s C-termini with archaeal 20S proteasomes and implications for the eukaryotic proteasome-ATPase interactions. EMBO J. 29, 692–702 (2010).

23. Yao, Y. et al. Structural and functional characterizations of the proteasome-activating protein PA26 from Trypanosoma brucei. J. Biol. Chem. 274, 33921–33930 (1999).

24. Hu, K. et al. Probing the cooperativity of Thermoplasma acidophilum proteasome core particle gating by NMR spectroscopy. Proc. Natl Acad. Sci. USA 114, E946–E954 (2017).

25. Adams, G. M. et al. Structural and functional effects of PA700 and modulator protein on proteasomes. J. Mol. Biol. 273, 646–657 (1997).

26. Barthelme, D., Chen, J. Z., Grabenstatter, J., Baker, T. A. & Sauer, R. T. Architecture and assembly of the archaeal Cdc48/20S proteasome. Proc. Natl Acad. Sci. USA 111, E1687–E1694 (2014).

27. Haselbach, D. et al. Long-range allosteric regulation of the human 26S proteasome by 20S proteasome-targeting cancer drugs. Nat. Commun. 8, 15578 (2017).

28. Kleijnen, M. F. et al. Stability of the proteasome can be regulated allosterically through engagement of its proteolytic active sites. Nat. Struct. Mol. Biol. 14, 1180–1188 (2007).

29. Osmulski, P. A., Hochstrasser, M. & Gaczynska, M. A tetrahedral transition state at the active sites of the 20S proteasome is coupled to opening of the alpha-ring channel. Structure 17, 1137–1149 (2009).

30. Ruschak, A. M. & Kay, L. E. Proteasome allostery as a population shift between interchanging conformers. Proc. Natl Acad. Sci. USA 109, E3454–E3462 (2012).

31. Ustrell, V., Hoffman, L., Pratt, G. & Rechsteiner, M. PA200, a nuclear proteasome activator involved in DNA repair. EMBO J. 21, 3516–3525 (2002).

32. Bai, L. et al. Structural analysis of the dodecameric proteasome activator PafE in Mycobacterium tuberculosis. Proc. Natl Acad. Sci. USA 113, E1993–E1992 (2016).

33. Hu, K. et al. Proteasome substrate capture and gate opening by the accessory factor PafE from Mycobacterium tuberculosis. J. Biol. Chem. 293, 4713–4723 (2018).

34. Benaroudj, N. & Goldberg, A. L. PAN, the proteasome-activating nucleotidease from archaeabacteria, is a protein-unfolding molecular chaperone. Nat. Cell. Biol. 2, 833–839 (2000).

35. Ohi, M., Li, Y., Cheng, Y. & Walz, T. Negative staining and image classification - powerful tools in modern electron microscopy. Biol. Proced. Online 6, 23–34 (2004).

36. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. Nat. Methods 14, 331–332 (2017).

37. Zhang, K. Getf: real-time CTF determination and correction. J. Struct. Biol. 193, 1–12 (2016).

38. Rosenthal, P. B. & Henderson, R. Optimal determination of particle orientation, absolute hand, and contrast loss in single-particle electron cryomicroscopy. J. Mol. Biol. 333, 721–745 (2003).

39. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).

40. Delano, W. L. Pymol: an open-source molecular graphic tool. CCP4 Newsl. Protein Crystallogr. 40, 82–92 (2002).

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Author contributions Z.Y. performed most of the biochemical and cryo-EM experiments and participated in exploratory research and analysis. J. Comput. Chem. 25, 1605–1612 (2004).

41. DeLano, W. L. Pymol: an open-source molecular graphic tool. CCP4 Newsl. Protein Crystallogr. 40, 82–92 (2002).

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