The YΦ motif defines the structure-activity relationships of human 20S proteasome activators

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The 20S proteasome (20S) facilitates turnover of most eukaryotic proteins. Substrate entry into the 20S first requires opening of gating loops through binding of HbYX motifs that are present at the C-termini of certain proteasome activators (PAs). The HbYX motif has been predominantly characterized in the archaeal 20S, whereas little is known about the sequence preferences of the human 20S (h20S). Here, we synthesize and screen ~120 HbYX-like peptides, revealing unexpected differences from the archaeal system and defining the h20S recognition sequence as the Y-F/Y (YΦ) motif. To gain further insight, we create a functional chimera of the optimized sequence, NLSYYT, fused to the model activator, PA26E102A. A cryo-EM structure of PA26E102A-h20S is used to identify key interactions, including non-canonical contacts and gate-opening mechanisms. Finally, we demonstrate that the YΦ sequence preferences are tuned by valency, allowing multivalent PAs to sample greater sequence space. These results expand the model for termini-mediated gating and provide a template for the design of h20S activators.
The proteasome is a critical regulator of protein homeostasis that degrades ~90% of all eukaryotic proteins\(^1\). The enzymatic activity of this system is carried out by the 20S proteasome (20S), a cylindrical, complex composed of four stacked rings enclosing an axial channel. To be degraded, potential substrates must first diffuse through a pore at the center of the distal α-rings before encountering the peptidase sites within the inner β-rings. N-terminal extensions of the α-subunits gate entry into the pore, limiting the degradation of bystander proteins\(^2\). In turn, this barrier creates a key regulatory role for the proteasome activators (PAs)\(^3\)–\(^5\), large particles that bind the 20S, open the gates, and facilitate substrate selection and entry. One evolutionarily conserved\(^6\)–\(^8\) way that PAs achieve this goal is by using a tripeptide motif at their extreme C-termini, which is characterized by a hydrophobic amino acid, followed by a tyrosine and then any amino acid (HbYX). HbYX motifs open the gates by docking into pockets located between adjacent α-subunits of the 20S (termed α-pockets) (Fig. 1a)\(^9\)–\(^11\). Unlocking the details of HbYX recognition at this protein-protein interaction (PPI) will deepen our understanding of the gate opening mechanism and enable novel strategies to regulate protein degradation in cells.

The HbYX model for gate opening was pioneered from studies by Goldberg and colleagues using the C-terminal sequence of the archaeal PA, proteasome-activating nucleotide (PAN)\(^9\). This work, and subsequent structures, revealed key roles for the penultimate Tyr residue and the terminal carboxylate of the HbYX motif\(^12,13\). However, unlike the archael system, the human 20S proteasome (h20S) is a hetero-oligomer, containing seven distinct α-pockets for binding to the HbYX motif. Thus, it is not clear whether the same structure-activity relationships (SAR) that govern the recognition of the HbYX motif in the archaeal system are conserved in the h20S. Addressing this question in the context of natural PAs has been challenging. For example, the structural topology of PA700/19 S, the eukaryotic homolog to PAN, means that its six distinct C-termini are not allowed to sample different α-pockets\(^14\). Moreover, there are confounding effects of cooperativity, ATP hydrolysis\(^15\)–\(^17\), and contributions from the additional subunits of PA700\(^18\)–\(^20\). We envisioned that one way to circumvent these issues and reveal the underlying SAR might be to use synthetic peptides instead of native PAs. Indeed, HbYX-containing peptides derived from the C-termini of the human Rpt subunits of PA700, have been shown to bind the α-pockets and stimulate turnover of substrates by the 20S\(^9,21,22\). Inspired by that approach, we hypothesized that a library of peptides could be used to understand the SAR of the HbYX motif in the h20S. Importantly, beyond the important insights into the molecular mechanisms of h20S gate opening, such investigations might be expected to inform the design of small molecules that bind the α-pockets\(^23\).

In this study, we design and synthesize ~120 peptides derived from Rpt5’s C-terminus and evaluate their ability to stimulate the peptidase activity of the h20S in vitro. This analysis reveals sequence preferences that differ from the canonical HbYX motif as derived in archaea. We refer to this re-defined preference as the YΦ motif. To better understand the structural underpinnings of the YΦ motif, we draft an optimal sequence, NLSSYT, to the C-termini of an inert PA platform and solve a 2.9 Å resolution structure of the PA bound to the h20S by cryo-electron microscopy (cryo-EM). Remarkably, the same orientation of NLS5YT is observed in five of the seven α-pockets, suggesting a conserved set of molecular interactions. Analysis of the bound YΦ motif reveals specific inter- and intramolecular contacts, which are involved in molecular recognition and gate opening. Finally, using a series of chimeras, we find that the valence of the PA displaying the YΦ motif (e.g. monomer vs. heptamer) tunes these sequence preferences, with multivalent PAs able to overcome otherwise non-ideal sequences. Together, these studies reveal mechanisms of termini-dependent gate opening in the h20S and establish a consensus sequence for monovalent activators.

**Results**

**Nomenclature of the HbYX motif and α-pockets.** Throughout this work, we will refer to the carboxy-terminal residue of the HbYX motif as termed P1, the next residue P2, etc. In this parlance, the canonical HbYX motif is defined as having a hydrophobic residue at P3, a preference for Tyr at P2, and any residue at P1. Another key part of the HbYX motif is that it contains the terminal carboxylate at the P1 position, which forms a critical salt bridge with a conserved cationic side chain at the base of the α-pocket; for example a lys66 of the Thermoplasma acidophilum 20S (Fig. 1a)\(^9\). Unless otherwise noted, we will use the residue numbering of the T. acidophilum 20S.

**Establishing a pipeline for measuring peptide-mediated activation of the h20S.** To determine the SAR of the HbYX motif in the h20S system, we synthesized, characterized, and assayed a library of peptides derived from the C-terminus of human Rpt5 with the native sequence: ANLQYYA. For each peptide, its capacity to accelerate substrate turnover by purified h20S was monitored by the hydrolysis of a fluorogenic substrate (suc-LLVY-amc). Historically, peptide-based activators have been shown to exhibit atypical dose responses, including partial inhibition of the 20S at higher concentrations\(^24\). Similarly, sodium dodecyl sulfate (SDS) and other detergent-like small molecules have been reported to activate the 20S nonspecifically; these otherwise useful solubilizing agent are typically excluded from buffers in proteasome activity assays\(^25\).

With these considerations in mind, we first optimized the buffer conditions and establish a triage pipeline to remove non-specific activators. These efforts were inspired by previous reports to identify buffers in which the difference between the 20S activities in the basal and stimulated conditions are maximized\(^26,27\). Using this starting condition (see Methods), we observed that high concentrations of either peptide or known small-molecule activators would inhibit, rather than stimulate, substrate turnover by the h20S. We reasoned that this effect was likely due to aggregation at the higher concentrations\(^28\).

Supplementing the assay buffer with a non-ionic surfactant (0.01% Pluronic F-68 *) attenuated the loss in dose-responsiveness caused by high concentrations of activators (Supplementary Fig. 1a–c). Yet, we also identified peptides displaying atypical dose responses that could not be resolved with the solubilizing agent (e.g., Cys- and Met-containing peptides) (Supplementary Fig. 1d) and these peptides were therefore excluded from further study. Peptides that yielded visibly cloudy solutions at 10 mM in DMSO were also omitted (Supplementary Fig. 1e). Finally, the remaining peptides were evaluated for solubility by dynamic light scattering (DLS) (see Methods) (Supplementary Table 1). Together, these triage steps restricted the sequence space that could be studied, but also minimized contributions from non-specific mechanisms.

**Structure-activity relationships (SAR) of hRpt5 peptides deviate from the HbYX model.** In the first series of peptides, we performed an alanine mutational scan of hRpt5: ANLQYYA. Consistent with previous reports\(^29\), the parent peptide (termed wildtype or WT) stimulated turnover of suc-LLVY-amc by ~3x (Fig. 1b). An EC\(_{50}\) value could not be determined, due to relatively weak potency, so, at this stage, we compared peptides based on their relative ability to stimulate hydrolysis rate. Replacing
either the P3 ('Hb') or P2 ('Y') positions with Ala completely ablated activity, in agreement with the HbYX model. Surprisingly, replacing the P4, P5 or P6 residues with Ala also had appreciable effects, including an unexpected enhancement in activity by Ala substitution at P4 (1.3× over WT), complete ablation of activity at P5, and modest impairment at P6 (~80% of WT) (Fig. 1b). A series of N-terminal truncations revealed that the 6- and 7-mer peptides were significantly more stimulatory than shorter ones (Fig. 1c), supporting a role for residues upstream of the tripeptide motif.

Guided by these findings, we designed a series of hexapeptides to probe the SAR in more detail. Peptides were synthesized and N-terminally acetylated (Ac), which generally enhanced activity over the free amine (Supplementary Fig. 2a). Acetylated peptides were then assessed at a single dose (250 µM) against the h20S to obtain an initial overview of their relative activities. In the first set
of comparisons, we varied the P1, P2, and P3 positions, replacing the wild-type residue with chemically and/or structurally diverse amino acids. We found that the h20S had sequence preferences at the P1 position, with Thr favored over other residues that were tested. More importantly, Tyr seemed to be exclusively required at the P3 position; such that even other hydrophobic residues, including Leu and Ile, were not tolerated. Lastly, either Phe or Tyr was preferred at the P2 position (Fig. 1d). When we compared these sequence preferences to those reported for the archaeal 20S, we found the h20S had both similarities and significant differences (Supplementary Fig. 3).

We were particularly intrigued by the gain in activity caused by Ala at P4 (see Fig. 1b), so we generated a focused series of P4-substituted hexapeptides to explore this position further. In this collection, the P1 was uniformly replaced with Thr, which we found to improve aqueous solubility and activate better than Ala, allowing us to obtain saturable stimulation curves and calculate EC50 values (Supplementary Fig. 4a). We found that none of the P4 substitutions completely ablated activity, suggesting that, unlike P2 or P3, the requirements at the P4 residue are more permissive. Nonetheless, modifications at P4 impart the greatest modulatory effect on the rate of hydrolysis (up to 3× over WT), with positively charged or bulky side chains generally exhibiting lesser stimulation (Fig. 1e). Additionally, residues known to disrupt α-helical character (e.g., Gly and Pro) were well-tolerated at the P4 position, consistent with previous observations. Lastly, we explored the contributions of the P5 and P6 position. Even though we had previously found that truncations of these residues diminished activity, residues substitutions at P5 or P6 had modest effects (see Supplementary Fig. 2b–d), suggesting that the side chain identity was less important in these positions. Next, we incorporated the optimal substitutions at both P1 and P4 to probe whether they are synergistic. In these experiments, we were particularly interested in understanding which substitutions might enhance the apparent EC50 (as a pseudo approximation of affinity) and which ones might impact the rate of hydrolysis. The results showed that Thr at P1 improved only the EC50, while Ser at P4 enhanced both EC50 and the rate of hydrolysis. Combining both substitutions had an additive effect (Fig. 1f), yielding an optimized sequence Ac-NLGYYT (EC50 = 35.5 ± 1.1 μM). For brevity, we refer to this sequence as Opt5.

Based on our peptide study, the penultimate tyrosine residue is not essential for activating the h20S. Previous work had demonstrated that certain HbYX-containing PAs can function with a Phe or Tyr (F/Y—denoted as Φ) at P2, however, the predicted importance of the Tyr at the P3 position seemed unique. To test this idea, we substituted either the P2 or P3 Tyr residues of Opt5 (referred to here as Opt5ΦY for clarity) with Phe (Opt5ΩY or Opt5ΦY, respectively) and measured the ability of these peptides to stimulate h20S. Removal of the P3 hydroxyl (Opt5ΦY) dramatically reduced activity by ~70%, while loss of the P2 hydroxyl (Opt5ΩY) had no appreciable effect on turnover and actually improved potency by ~2-fold relative to Opt5YY (EC50 = 17.3 ± 1.1 μM) (Fig. 1f). Previous work had also suggested that a Phe at P2 might be favored, so we assessed the generality of this finding by testing two additional peptide sequences (Ac-NLSYΦA and Ac-NLGYΦT), noting up to a 3-fold improvement in potency by Phe over Tyr in both cases (Supplementary Fig. 4b).

To this point, measurements of proteasome activity were restricted to the suc-LLVY-amc probe, which measures chymotryptic-like activity. If the optimized peptide Opt5 induces gate opening, we would expect that it would also stimulate the tryptic-like activity. Indeed, we found that treatment with Opt5 and other peptides promoted the tryptic-like activity of the h20S, as measured using boc-LRR-amc (Supplementary Fig. 5a). Moreover, these peptides also stimulated hydrolysis of a longer, nonapeptide substrate (FAM-LFP), which is known to require gate opening for its entry into the proteasome (Supplementary Fig. 5b). Taken together, these findings suggest that h20S activation by hRpt5-like peptides occurs through interactions with at least the last four residues, with additional contributions from P5 and P6. The sequence requirements are expanded from the HbYX motif and are summarized as the ΦΦ motif (see below).

**PA26E102A-Opt5 induces terminus-dependent gate opening of the h20S.** To understand the molecular basis for the sequence preferences in the ΦΦ motif, we attempted to determine the structure of h20S bound to Opt5 peptides by cryo-EM but were unable to obtain high-resolution structures. Rather, inspired by the previous studies, we sought to use the homoeptamer activator PA26 as a scaffold for the display of multiple copies of Opt5 (NLSYΦY). Instead of C-terminal HbYX motifs, PA26 has an activation loop that displaces the N-terminal gating residues through direct contacts with the reverse-turn loop at αPro17 in the α-ring (Fig. 2a). An Ala substitution in the activation loop (E102A) renders PA26 inactive. Previous studies showed that the capacity of the disabled-loop mutant (PA26E102A) to induce gate opening was rescued by grafting PAN’s HbYX motif in place of the native C-termini. Thus, we envisioned likewise using PA26E102A to enable high-resolution structural studies of termini-dependent gate opening. Archael PA26 is capable of activating the h20S, however, it remains unclear whether the E102A mutation impairs this function, as has been reported for the archaeal 20S. Using the T. brucei PA26, we verified that PA26E102A was unable to stimulate the h20S. Moreover, PA26E102A’s ability to bind h20S was significantly diminished, as measured by biolayer interferometry (BLI) (Fig. 2b). Next, we genetically installed NLSYΦY in place of the last six native residues of PA26 (PA26E102A-Opt5)
to generate a PA that now bound the h20S \((K_d = 360 \pm 160 \text{ pM})\) and potently stimulated its activity \((EC_{50} = 42.1 \pm 1.2 \text{ nM})\) via its C-terminus (Fig. 2b). To probe how the binding of Opt5 activates h20S, we then determined the structure of the h20S-PA26\(_{E102A,\text{Opt5}}\) complex by cryo-EM to an overall resolution of 2.9 Å (Supplementary Figs. 6 & 7 and Supplementary Table 2). The cryo-EM structure resolves a single particle of the PA26\(_{E102A,\text{Opt5}}\) heptamer bound to an α-ring of the h20S. At this site, the N-terminal extensions of the α-subunits were displaced from the central pore (Fig. 2c) and the diameter of the pore was widened by 3.8 Å relative to the apo α-ring (Supplementary Table 3), reminiscent of other open gate structures\(^8\). Consistent with findings from an analogous study with an archaeal 20S, binding of PA26\(_{E102A,\text{Opt5}}\) at one α-ring of the h20S did not allosterically open the distal α-ring gate\(^2\). Cryo-EM resolves open gate conformation of the h20S. Using this structure, we explored the potential mechanisms contributing to the opening of the h20S gate. In other systems, the open gate conformation is regulated by a cluster of conserved N-terminal residues (αTyr8, αAsp9, αPro17, and αTyr26)\(^7,8\). Specifically, these residues are repositioned away from the central pore during gate opening and are anchored in that position by a characteristic set of intra- and intermolecular contacts. However, these residues are less conserved in humans, particularly in α1 and α2 (α-subunits are labeled to match the numbering used in yeast 20S), where the canonical αTyr8 and αAsp9 are replaced with α1 Phe9 and α2 Ser7, respectively. We wondered whether these differences might impact either the gating mechanism or the extent of opening. In the liganded α-ring of our structure, the N-termini of α5, α6, and α7 formed the expected, ordered clusters at the α-subunit interface, including a repositioned αPro17 within CH-π distance from αTyr26 and αTyr8, consistent with the fully open state\(^19,20\). However, the equivalent residues in α1 and α2 formed clusters with fewer contacts likely resulting from displaced polar contacts in the noncanonical α1 Phe9 and α2 Ser7 residues, respectively (Supplementary Fig. 8a). In addition, the N-terminal residues were poorly resolved in α1 and α2 relative to N-termini from the ordered clusters. Interestingly, we noted that although subunits neighboring α1 and α2, namely α3 and α4, have canonical N-terminal sequences, these conserved residues formed limited contacts within clusters and/or had side chains that could not be structurally resolved. Furthermore, α2, α3, and α4, the subunits that primarily seal the pore’s entrance of the closed gate apo h20S, displayed disordered N-terminal extensions that seems to partially occlude the pore, unlike the ordered and fully displaced N-termini of reported open states (Supplementary Fig. 8b). Such conformations have uniquely been observed in human 26 S proteasome structures\(^16,17\), however, it was not entirely clear if the 19S’s C-termini were incapable of fully opening the gate. These observations support previous claims\(^30\) and further demonstrate how noncanonical residues in the human α-subunits limit gate opening by displacing stabilizing interactions, which propagates disorder along the α-ring to, in turn, destabilize a fully opened gate state in the h20S. To discern the conformational changes associated with gate opening, we aligned the β-rings of our structure to that of apo h20S (PDB ID: 4R3O)\(^34\) because the β-subunits remain relatively unchanged during activation\(^1,13\). We observed that the Ca atoms of all seven αPro17 residues were radially displaced between 1.0 and 3.7 Å during gate opening. Consistent with the analyses of the N-terminal gate positions, the partially opened α1 and α2 N-termini had the smallest shifts in αPro17 (1.0 and 1.3 Å, respectively) (Supplementary Fig. 8c). The modest displacement of α2 Pro15 contrasts to the relatively large shifts typically observed by the N-terminus of the yeast α2 subunit\(^30\). This difference between yeast and human is interesting to note because both species share a noncanonical Ser in α2; thus, it is possible that replacement of αTyr8 with α1 Phe9, which is unique to h20S, is more restrictive of the fully open conformation. We next examined how the termini might be inducing gate opening in our structure. First, we confirmed that the disabled activation loop of PA26\(_{E102A,\text{Opt5}}\) does not interact with αPro17, suggesting that this structure allows the determination of termini-specific mechanisms, as intended. At the occupied α-pockets, most of the P2 and P3 Tyr side chains of Opt5 made H-bond contacts with residues located in the reverse turn between αPro17 and helix 0 (Supplementary Fig. 9a). This finding suggests that residues of Opt5 act directly on the reverse-turn loop to open the gate. We also detected that α-subunits were rotated an average of 1.5 ± 0.4° about the axial channel (Supplementary Fig. 9b) and observed significant side chain rearrangements throughout the α-ring (Fig. 3a), which were induced by PA26\(_{E102A,\text{Opt5}}\). Indeed, both rigid body rotations and induced-fit conformational changes have been attributed to termini-dependent gate opening\(^12,13,17,32\).
Overall, we conclude that termini-mediated activation of the h20S largely proceeds through the conserved gating mechanism observed in other proteasomes; but with distinctions in the asymmetric gate opening of the α-rings, potentially linked to the noncanonical α1 Phe9.

Opt5-bound α-pockets reveal key contacts for terminus-dependent h20S activation. We next examined the direct interactions of Opt5 in the h20S α-pockets. This analysis was aided by the matched stoichiometry of C-termini to α-pockets, together with the fact that the α-pockets were largely resolved to better than 2.9 Å resolution (see Supplementary Fig. 7d). Six of the seven α-pockets contained well-defined density corresponding to the termini of PA26E102A-Opt5. As previously predicted,8,20,35 the α7/α1-pocket (interfacing the α7 and α1 subunits) did not contain density for Opt5, likely because this pocket does not have a canonical Lys residue (α1 His68). Of the remaining six sites, the termini in the α1/α2-pocket had a noticeably distinct structure. Specifically, its terminal carboxylate was displaced (5.1 Å) from the ε-amino group of α1 Lys63 and therefore unable to form the expected salt bridge, which has previously been shown to be critical for binding of native PAs and HbYX peptides.9,12. To verify the importance of the negatively charged terminal carboxylate to overall h20S stimulation, we amicidated the C-terminus of Opt5 peptide and confirmed that it lost all activity in turnover assays (see Fig. 1f). Hence, we categorized the C-termini in the α2/α3, α3/α4, α4/α5, α5/α6 and α6/α7 pockets as binding in an anchored (A) state and the C-termini in the α1/α2-pocket as binding in the unanchored (U) state (Fig. 3a, b).

The peptide backbone of Opt5, of both A and U states, were well-resolved for the P1, P2, P3, and P4 positions, enabling further assessment. First, we noticed that the backbone conformations of the bound C-termini are different between the A and U states. The dihedral angles of the P2-P3 residues of the bound Opt5 form a β-strand structure for the A state but a right-handed α-helix for the U state (Fig. 3c). In a recent study of the archaeal 20S-PAN complex, the terminal residues of PAN in PA26E102A-PAN adopt α-helices when docked into α-pockets of the T. acidophilum 20S52, suggesting that the β-strand conformation might be specific to eukaryotic 20S proteasomes and/or ΥΦ motifs. Next, we noticed that the five C-termini that bound in the A state are nearly identical; each predicted to make between 20 and 25 H-bonds in the α-pocket, including the critical salt bridge (Fig. 3a). In contrast, there are fewer contacts (3 H-bonds and no salt bridge) made between the U state peptide and the α1/α2-pocket. Another distinction between the U and A states is the configuration of predicted H-bonds between Opt5 and residues in the α-pocket, most notably, αGlu25. In the U state, H-bonding occurs between the carboxyl side chain of α1 Glu26 and the hydroxyl of the P2 Tyr. In the A state, hydrogen
bonding is mediated between aGlu25 and the P3 Tyr side chain, which allows for additional contacts to the α-pocket by the hydroxyl group of the P2 Tyr (i.e. a2/a3, a4/a5, & a5/a6) (Fig. 3b). The aGlu25 is highly conserved from T. acidophilum to humans, including across all seven distinct α-subunits (see Supplementary Fig. 8a), further highlighting the potential importance of this H-bond contact. Together, these studies suggest that polar contacts within the α-pocket, notably H-bonding between the P3 Tyr and aGlu25, are involved in the termini-mediated gate opening of the h20S.

Intramolecular stacking of P3 and P2 residues regulates α-pocket engagement. Considering the heterogeneity of the α-pockets in the h20S, we were struck by how the C-terminals adopted strikingly similar orientations. For example, when we overlay the six bound C-terminals from the PA26E102A-Opt5-h20S structure, the P2 and P3 Tyr residues adopt a nearly identical, stacked orientation (see Fig. 3c). Moreover, we noted that of the subset of reported structures of eukaryotic PA-20S complexes, those with C-terminal ΨΦ motifs also depicted stacking of the aromatic P2 and P3 residues. In addition, Rpt5 and PA200/Bm10 displayed comparable dihedral angles, which resembled the backbone configuration of Opt5 to suggest that the distinct positioning of the Tyr residues may be a common feature of ΨΦ motifs (Supplementary Fig. 10). We next noted how the average distance (R) between the centroids of the P2 and P3 Tyr rings (4.5 ± 0.2 Å) and the average angle (θ) between the rings’ planes (26.8 ± 3.3°) closely matched energy-minimized calculations for a Tyr-Tyr dipeptide adopting an off-centered parallel, π stack (Fig. 4a)37. Thus, we postulated that intramolecular π stacking facilitates termini-gate opening by orienting Opt5 to a competent conformation within the α-pocket. To test this hypothesis, we explored how perturbations to the putative π stack affected the ability for peptide analogs of Opt5Ψ to stimulate h20S activity (Fig. 4b). In dose-response assays, replacement of the Phe side chain with a cyclohexyl group (4) at the P2 position, which should no longer π stack with the P3 Tyr side chain, reduced activity by 80% relative to Opt5Ψ, (Opt5Ψ4; EC₅₀ > 600 µM) (Fig. 4c). Likewise, an inversion of the aromatic quadrupole through a pentafluorophenylalanine (5) substitution at the P2 position (Opt5Y5) significantly reduced potency (EC₅₀ > 600 µM) and activity (by 80%) relative to Opt5Ψ. Less drastic perturbation of the π electron density, through the installation of ortho- or meta-chloro groups (2 and 3; Fig. 4b), had intermediate consequences (Fig. 4c). Although we cannot rule out steric effects, these experiments suggest that intramolecular π-stacking interactions between the Y and Φ residues contribute to Opt5 activity, perhaps by orienting the motif for engagement with aGlu25.

Valency tunes the sequence preferences for proteasomal activation. Native PAs and other 20S-binding partners are often multivalent, displaying multiple C-termini from a central scaffold. One effect of this valency is that binding avidity is increased, likely to promote cellular assembly of proteasome complexes. However, a less appreciated effect of valency in biological recognition is that it also tunes specificity, amplifying some preferences and allowing more variability in others. To determine if valency influences the sequence preferences of C-terminal recognition by the h20S, we generated and tested additional chimeras of the heptavalent PA26ΨE102Y, emphasizing on modifications at the P2 and P3 positions because of their aforementioned contributions. Unlike the monovalent peptides, Phe substitution of the P3 Tyr did not affect PA26ΨE102Y capacity to stimulate the h20S and minimally altered its potency (PA26ΨY & PA26Ψ; EC₅₀ = 42.1 ± 1.2 & 33.7 ± 1.2 nM, respectively). Similar to the trends observed with Opt5 peptides, Phe substitution of the P2 Tyr slightly improved the EC₅₀ (PA26ΨF; 23.5 ± 1.2 nM) (Fig. 5a). Interestingly, the binding affinity for h20S remain relatively unchanged when either the P2 or P3 Tyr residues were replaced with Phe. We also noted that PA26ΨY has a k_on that is ~3x slower than that of PA26ΨF and PA26Ψ, and yet, the calculated K_d for PA26ΨF remains within error of the others, owing to its significantly slower k_off (Supplementary Fig. 11b). We then generated an alternate, monovalent PA by grafting peptide sequences of interest onto the C-terminus of maltose-binding protein (MBP). Tethering the Opt5 sequence (NLSYYT) to the monovalent MBP, we found that the Opt5 chimera (MBPΨY) equipotently stimulated h20S (EC₅₀ = 41.8 ± 1.2 µM) relative to Opt5Ψ peptide. Likewise, the P2 Phe mutant was more potent (MBPΨF; EC₅₀ = 10.0 ± 1.2 µM), mirroring the SAR from the monovalent peptides. The P3 Phe mutant exhibited decreased activity (MBPΨY; EC₅₀ > 400 µM), however, the activity of MBPΨF was relatively diminished compared to its peptide equivalent (Fig. 5b). Together, we conclude that valency appears to the h20S to be more permissive of missing contacts in the PA’s C-termini, whereas monovalent ones largely adhere to the ΨΦ motif.

Next, we interrogated the impact valency might have on the relative importance of intramolecular π stacking by generating P3 or P2 Ala mutations in all three PA-types: monovalent peptide, monovalent MBP, and multivalent PA26ΨE102A. In stimulation assays, Ala mutations at either P3 or P2 completely inactivated both PA26ΨE102A and Opt5Ψ for adjacent P2 and P3 tyrosine residues37. Values are of bound Opt5 from the cryo-EM structure (n = 6) and reported as mean ± s.d. See Supplementary Table 3 for individual measurements. b Structure of the side chains from unnatural amino acids used in this study. c P2 modifications to the π electron density decreases the stimulatory effect of Opt5. Data are normalized to Opt5Ψ and plotted individually (n = 2 to 4). Reported EC₅₀ is a mean of EC₅₀ values calculated from two to four independent experiments with error reported as s.e.m.
amino acids, such as Ser, Thr, and Asp, were also relatively bulkier residues due to potential steric clashes with a neighboring side chain. Tentatively, small, aliphatic residues might be preferred over larger ones.

In contrast, the structural rationale for the preference at P4 is less clear. Pioneering studies of the HbYX motif in the archaeal system (Fig. 6a) have contributed significantly to our understanding of 20S activation. Herein, we aimed to deepen that understanding by characterizing how Rpt5-derived peptides stimulate the h20S. This effort uncovered narrower preferences for the P1 'X' and P3 'hydrophobic' residues and revealed an unanticipated role for the P4 position, suggesting that the h20S exhibits distinct sequence preferences. Although only a subset of amino acids could be explored because of solubility criteria, only Ala and Thr were strongly preferred at P1, a result that contrasts with the relatively broad specificity exhibited in the archaeal system (see Supplementary Fig. 3).

A potential reason for this preference was revealed by structural studies, which showed that Thr in the P1 position engages in H-bonding within h20S α-pockets. In contrast, the structural rationale for the preference at P4 is less clear. Tentatively, small, aliphatic residues might be preferred over bulkier residues due to potential steric clashes with a neighboring β-strand-turn in the α-pockets. In addition, a subset of polar amino acids, such as Ser, Thr, and Asp, were also relatively preferred at P4 (see Fig. 1e), indicating that H-bonding in that region may also be involved. However, further work is needed to elucidate the importance of these interactions, including whether they contribute to binding, gate opening or both.

In our h20S-PA26E102A-Opt5 structure, we noted that the C-termini bind in two different states (A and U). Although we concluded that PA valence influences which interactions mediate proteasome activation, we speculate that the A and U states might represent putative docking poses for Opt5Y peptides occupying α-pockets of the h20S, providing potential mechanistic insight into peptide-mediated gate opening. For instance, to explain the unprecedented contribution of the P3 Tyr in peptide/monomeric PAAs we conjectured that Opt5FY likely binds the P3 Tyr with a Phe residue. Altogether, this would suggest that H-bond formation between the P3 Tyr and αGlu25 acts as an anchor that promotes the Φ motif in the open gate competent A state. In support of this model, reported structures of both yeast26 and human26 S proteasomes, the P3 & P6 residues are likely to play a role. Note that multivalency seems to allow PAs to, in some cases, overcome a subset of these requirements.

**Discussion**

Pioneering studies of the HbYX motif in the archaeal system (Fig. 6a) have contributed significantly to our understanding of 20S activation. Herein, we aimed to deepen that understanding by characterizing how Rpt5-derived peptides stimulate the h20S. This effort uncovered narrower preferences for the P1 'X' and P3 'hydrophobic' residues and revealed an unanticipated role for the P4 position, suggesting that the h20S exhibits distinct sequence preferences. Although only a subset of amino acids could be explored because of solubility criteria, only Ala and Thr were strongly preferred at P1, a result that contrasts with the relatively broad specificity exhibited in the archaeal system (see Supplementary Fig. 3).

A potential reason for this preference was revealed by structural studies, which showed that Thr in the P1 position engages in H-bonding within h20S α-pockets. In contrast, the structural rationale for the preference at P4 is less clear. Tentatively, small, aliphatic residues might be preferred over bulkier residues due to potential steric clashes with a neighboring β-strand-turn in the α-pockets. In addition, a subset of polar amino acids, such as Ser, Thr, and Asp, were also relatively preferred at P4 (see Fig. 1e), indicating that H-bonding in that region may also be involved. However, further work is needed to elucidate the importance of these interactions, including whether they contribute to binding, gate opening or both.

In our h20S-PA26E102A-Opt5 structure, we noted that the C-termini bind in two different states (A and U). Although we concluded that PA valence influences which interactions mediate proteasome activation, we speculate that the A and U states might represent putative docking poses for Opt5Y peptides occupying α-pockets of the h20S, providing potential mechanistic insight into peptide-mediated gate opening. For instance, to explain the unprecedented contribution of the P3 Tyr in peptide/monomeric PAAs we conjectured that Opt5FY likely binds the h20S in the U state. Hence, we postulate that the diminished activity of Opt5FY is ultimately mediated by displacement of the salt bridge, which is attributed to fewer polar interactions within the α-pocket, namely the displaced H-bond caused by replacing the P3 Tyr with a Phe residue. Altogether, this would suggest that H-bond formation between the P3 Tyr and αGlu25 acts as an anchor that promotes the Φ motif in the open gate competent A state. In support of this model, reported structures of both yeast26 and human26 S proteasomes, the P3 & P6 residues of the Rpt3 subunit (F & Y, respectively), were strongly preferred at P1, a result that contrasts with the relatively broad specificity exhibited in the archaeal system (see Supplementary Fig. 4).
according to sequence preferences of the HbYX (orange) or nY acid residues at the P1, P2, P3, and P4 (Y only) positions are colored Supplementary Table 4. Each residue represents the relative frequency. The complete entry list in supplementary Table 4.

Fig. 7 The YΦ motif is strictly conserved in the eukaryotic, monovalent activator PA200. a–e Weblogo (http://weblogo.berkeley.edu) (48) representation of the last six residues of (a) PA200 (n = 7), (b) Rpt5 (n = 9), (c) Rpt3 (n = 9), (d) Rpt2 (n = 9), and (e) PAN (n = 41). Amino acid residues at the P1, P2, P3, and P4 (Y only) positions are colored according to sequence preferences of the HbYX (orange) or nYΦn (blue) motifs. Unspecified, along with P5 and P6 residues are in gray. The height of each residue represents the relative frequency. The complete entry list in Supplementary Table 4.

respectively), depict displaced salt bridges and C-termini that adopt α-helical turns, as observed in the U state. Moreover, Rpt3 is the least stimulatory of the Rpts with HbYX motifs21,22, further suggesting that interactions observed in our structure of the heptavalent PA26E102A-Opt5 could apply to binding of and activation by Opt5 peptides. However, it is plausible that the U state may simply be a binding pose unique to the Opt5 peptide, while the residues at the remaining positions seemed arbitrary (Fig. 7b). Unlike PA700, PAN is homo-oligomeric and so no individual monomer (e.g. Rpt5) is predicted to make an outsized contribution to overall binding or stimulation. Thus, in the case of PAN, valency seems to largely override the requirements of both the YΦ motif and the more permissive HbYX motifs. Nonetheless, the YΦ motif on the canonical HbYX model was similarly noted in an examination of another hexavalent regulator of the archaeal 20S, Cdc4839. Together, these observations suggest that C-terminal sequence identity, plus a contribution from valency, combine to dictate the PPIs between PAs and the proteasome. Further analysis of the SAR at these PPIs is important because additional binding partners of the 20S, with HbYX-like motifs of varying sequences and valences, have been identified in eukaryotes, but the full extent to which they regulate the proteasome remains limited59,42–44. Hence, it may be useful to create sub-categories of HbYX-like models, in which the valency of the PA is a key, defining characteristic.

Loss of proteasome function is implicated in many devastating proteinopathies, including neurodegenerative disorders45. Consistent with this idea, boosting 20S activity, by introducing either native46 or engineered activators47, has been found to be protective in cell-based disease models. These observations have motivated campaigns to discover drug-like molecules that mimic the activity of PAs43. This current work could be important in that effort, by defining the SAR associated with h20S α-pockets and providing a template for the rational design of pharmacological proteasome activators48,49. For example, we suspect that the arrangement of the YΦ motif, through π stacking, minimizes the entropic costs of binding for Opt5. For molecules that conform to this pharmacophore model, potency should be improved by rigidification of the biospecific equivalent to the Tyr-Tyr π stack. We also found that Opt5 docked into 6 of the 7 available α-pockets with remarkable similar structural states. This consensus structure might be a good starting point for understanding the requisite occupancy and binding interactions for inducing gate opening with a small molecule. Thus, the current work provides a potential blueprint for designing novel proteasome activators, while also extending our knowledge of the mechanisms of h20S activation.

Methods
Reagents. Human 20S proteasome was purchased from the Proteasome Center. pET28 6His PA26 (49 V) cloning vector was a gift from Philip Cof. pET 6His MBP TEV LIC cloning vector (1 M) was a gift from Scott Gradia (Addgene plasmid # 29656).

Strains and Plasmids. The E. coli strain Top10 was used for propagating plasmids. BL21 (DE3) cells were used for the expression and purification of recombinant proteins. Primers are listed in Supplementary Table 5.

Peptide synthesis. Peptides were synthesized by Fmoc solid-phase peptide synthesis on a Syro II peptide synthesizer (Biotage) at ambient temperature and atmosphere on a 12.5 μM using either pre-loaded Wang resin or Rink amide resin (Sigma-Aldrich). Coupling reactions were run with 4.9 eq. of HCTU (O-(1H-6-
chlorobenzo triazol e-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate), 5 eq. of Fmoc-AA-OH and 20 eq. of N-methylmorpholine (NMM) in 500 µL of N,N-
dimethylformamide (DMF). Fmoc-AA-OH was double coupled for 3 min while shaking for each position. Fmoc deprotection was conducted with 500 µl 40% 4-methylpyridine in DMF for 3 min, followed by 500 µl 20% 4-methylpyridine in DMF for 10 min and six washes with 500 µl of DMF for 3 min. Acetylation of the N-terminus was achieved by reacting 20 eq. acetic anhydride and 20 eq. N,N-di- 
propylethylamine (DPEA) in 1 mL in DMF for 2 h while shaking. Peptides were 
cleaved with 500 µl of cleavage solution (95% trifluoroacetic acid (TFA), 2.5% water and 2.5% triisopropylsilylane) while shaking for 2 h. Crude peptides were pre-
cipitated in 15 mL cold 1:1 diethyl ether: hexanes and air-dried overnight. Crude 
peptides were solubilized in a 1:1:1 mixture of DMF: water: acetonitrile, filtered, 
and purified by high-performance liquid chromatography (HPLC) on an Agilent 
Pursuit 5 C18 column (5 mm bead size, 150 x 2.1 mm) using Agilent PrepStar 
218 series preparative HPLC. The mobile phase consisted of A, 0.1% TFA in water and 
B, 0.1% TFA in acetonitrile. Peptides were purified to >95% homogeneity 
confirmed by liquid chromatography-mass spectrometry before solvent was 
removed by lyophilization. Peptides were resuspended in 1:1 water: acetonitrile, 
lyophilized again in tared tubes and stocks were stored at −20 °C.

**Dynamic light scattering (DLS).** From 10 mM stocks in DMSO, three 4-fold serial 
dilutions of each peptide were prepared in assay buffer that was 
filtration of each peptide before solvent was 
diluted to >95% homogeneity

**Protein Assay Kit (Thermo Scientific, 23225) and bovine serum albumin (BSA) as a standard.** 

**Biotinylation of h20S.** Commercially available h20S was dialyzed into PBS (Gibco Life 
Technologies pH 7.5 and biotinylated with 20 eq of NHS-Biotin (Thermo 
Scientific, 20217) at 4 °C for 2 h. Biotin-conjugated h20S was isolated from excess, 
unreacted NHS-Biotin with a PD-10 desalting column (Amersham Biosciences), 
and concentrated with a centrifugal filter concentrator (MilliporeSigma, UFC100). 
Aliquots of biotinylated h20S were snap-frozen and stored at −80 °C for later use.

**Binding kinetics analysis.** Bia-layer interferometry (BLI) data of PA26 activators were measured 
using an Octet RED384 (ForteBio). The reactions were carried out in black 384-well plates (Greiner Bio-One, 781209) at 25 °C with a volume of 85 µl 
per well in BLI buffer (assay buffer containing 0.2% (w/v) BSA (Sigma)). 
Biotyi-
lated h20S (30 nM) were immobilized on streptavidin (SA) biosensors. Serial 
dilutions of PA26 in BLI buffer were used as analyte. Association was observed by 
immersing loaded biosensors into solutions of analyte for 450 s. No binding 
was detected of the analyte to unloaded sensors. Dissociation was observed by trans-
ferring the sensor to a well-containing binding buffer and no analyte for 1200 s. 
Binding affinities (Kd) and kinetic parameters (kon and koff) were calculated from a 
[2.1 heterogeneous light scattering](Model of the data using the Octet data analysis software (Octet Data Analysis 7.1).

**Cryo-electron microscopy data collection and image processing.** Cryo-EM data 
were acquired using the Leginon 3.3 software for automated data acquisition51 
using a Titan Krios (Thermo Fisher) equipped with a K2 Summit (Gatan) direct 
electron detector in counting mode (Supplementary Table 2). Movies were 
collected by navigating to the center of a hole and image shifting a frame of –600 nm 
diameter to 10 targets situated at the periphery of the 2 µm hole. A total of 13,329 
movies were recorded at a nominal magnification of 29,000 × (1.03 Å pixel size) 
and composed of 29 frames (250 ms per frame, 50 e− Å−2 per frame). Movie collection was guided by real-time assessment of image quality using the 
Leginon 3.3 image processing environment.52 Frame alignment and dose 
weighting were performed in real-time using UCSF Motiคอน52. Estimation of the contrast transfer function (CTF) was performed on aligned, unweighted, 
micrographs using GIC.106 All data post-processing steps were conducted in RELION 3.0.56

**Proteasome activity assays.** Stimulation of h20S proteasome (Proteasome Center) 
was assayed in 384-well plates (Greiner Bio-One, 781209) using the fluorogenic 
substrate peptide succ-LLVY-amc (AnaSpec, AS-63982), boc-LRR-amc (AdipoGen, 
AG-CP-0014) or FAM-LFP (5-FAM-AKVYPYPMEK(QXL520)-NH2; AnaSpec) 
in assay buffer containing 50 mM Tris pH 7.5, 10 mM MgCl2, 200 µM adenosine 
triphosphate (ATP), 1 mM DTT, and 0.01% Pluronic F-68® (Gibco Life 
Technologies, 24040032) in a total volume of 30 µL. Human 20S (h20S; (final 
concentration of 4 nM) was incubated in the presence or absence of activators 
(0.2–600 µM, BPP 0.2–400 µM; PA26, 0.2–400 µM) for 30 min at room 
temperature for 5 min. Substrate succ-LLVY-amc (10 µM; boc-LRR-amc, 20 µM; FAM-LFP, 100 nM) was added immediately before reading. Fluorescence intensity of 
succ-LLVY-amc and boc-LRR-amc (excitation, 355 nm; emission, 440 nm; cutoff, 435 nm) or FAM-LFP (excitation, 490 nm, emission, 520 nm, cutoff, 515 nm) were 
measured by fluorometry over the course of the reaction (RFU per s). Data were processed and fit in GraphPad Prism 8.1.2. The baseline 
hydrolysis was normalized to the total mean activity for the lowest concentration of every 
activator in assay buffer in a given plate and expressed as a percentage of the 
reported. The normalized activity was plotted relative to log(agonist). Data was 
fit to the model for log(agonist) versus response (variable slope). In Eq. (1), 
Y = log[(activator, μM) except for PA26-based activators, which are in nanomolar.

![Figure 1](https://example.com/figure1.png)

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filtered to 60 Å and used to guide the initial 3D refinement and 3D classification. 288,515 particles corresponding to 3D classes without artefactual features were chosen for further data processing. To minimize the detrimental effects of pseudo-symmetry (C2) on resolution, the raw particles were C2 symmetry expanded, 3D refined, and a python script was used to determine the x- and y-shifts required to reposition the proteasome gate at the center of the particle box. The particles at the new center were re-extracted to serve as individual asymmetric units without down-sampling in a box of 256 x 256 x 256 pixels, resulting in a total of 577,030 particles. A round of reference-free 2D classification enabled us to remove the ends of h20S particles that lacked an activator molecule. This combined expansion and classification approach yielded 521,860 particles. We subjected these particles to 3D classification (k = 8) and selected 384,539 particles whose parent 3D classes resembled a fully assembled h20S-Pa26UFA0-Cy6 complex. Following removal of particle replicates with a python script, 326,676 particles were CTF and beam tilt refined. Further 3D classification without alignment (k = 10) revealed two classes with an unresolved alpha subunit helix. We attributed this finding to rotational misalignment around the C7 pseudo-symmetry, the longitudinal axis of the complex and discarded these particles. The remaining 247,362 particles were then sorted and pruned by z-score, yielding 254,960 particles for final 3D refinement (2.9 Å) (Supplementary Figs. 6 and 7).

**Atomic model building.** The atomic model was built by using the h20S and Pa26 (PDB IDs: 5AQQ and 1YAU, respectively) as templates and rigid body fitting each subunit with Chimera in the electron density map. Subunits for which there was no density as a result of the symmetry expansion and re-extraction processing approach, were removed from the template. The modified templates were subject to one cycle of morphing and simulated annealing in PHENIX 1.10.1, followed by a total of 5 real-space refinement macrocycles with atomic displacement parameters, secondary structure restraints, local grid searches, and global minimization. After PHENIX refinement, manual real-space refinement was performed in Coot 0.8.9.1. Multiple rounds of real-space refinement in PHENIX (five macrocycles, Ramachandran and rotamer restraints, no morphing, no simulated annealing) and Coot were performed to address geometric and steric discrepancies identified by the RCSB PDB validation server. To ensure atomic models were not overfit as a result of real-space refinement, map-to-model FSCs were calculated with PHENIX 1.10.12 (Supplementary Fig. 7b). All images were generated using UCSF Chimera 1.14 and PyMol 2.3.2.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The structural data generated in this study have been deposited in the Electron Microscopy Databank under EMD-22259 and in the Protein Databank (PDB) under 6XM1. The raw and processed data generated in this study are provided in the Source Data file. The reporting summary for this Article is available as a Supplementary Information file. All data supporting the findings of this study is available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions

K.A.O.-N. and J.E.G. designed the studies and wrote the manuscript. All authors edited the manuscript. K.A.O.-N., S.K.W., and N.C. conducted biochemical experiments, performed data analysis, and generated necessary reagents. A.H.P. performed cryo-EM sample preparation, data collection, and data processing. J.E.G., K.A.O.-N., A.S., and G.C.L. provided funding.

Competing interests

The authors declare no competing interests.

Additional information

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