

Proteasome allostery as a population shift between interchanging conformers

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Protein degradation plays a critical role in cellular homeostasis, in regulating the cell cycle, and in the generation of peptides that are used in the immune response. The 20S proteasome core particle (CP), a barrel-like structure consisting of four heptameric protein rings stacked axially on top of each other, is central to this process. CP function is controlled by activator complexes that bind 75 Å away from sites catalyzing proteolysis, and biochemical data are consistent with an allosteric mechanism by which binding is communicated to distal active sites. However, little structural evidence has emerged from the high-resolution images of the CP. Using methyl TROSY NMR spectroscopy, we demonstrate that in solution, the CP interconverts between multiple conformations whose relative populations are shifted on binding of the 11S activator or mutation of residues that contact activators. These conformers differ in contiguous regions of structure that connect activator binding to the CP active sites, and changes in their populations lead to differences in substrate proteolysis patterns. Moreover, various active site modifications result in conformational changes to the activator binding site by modulating the relative populations of these same CP conformers. This distribution is also affected by the binding of a small-molecule allosteric inhibitor of proteolysis, chloroquine, suggesting an important avenue in the development of therapeutics for proteasome inhibition.

NMR methyl TROSY | regulatory particle | allosteric network | proteasome inhibitors

The proteasome is a supramolecular machine that is central to protein degradation. The importance of the proteasome for cellular function is underscored by its role in the removal of misfolded or otherwise damaged proteins whose accumulation in the cell is cytotoxic, by its involvement in the immune response through the production of antigenic peptides, and by its regulation of the cell cycle through controlling the lifetime of key proteins that modulate gene expression (1). In addition, the proteasome serves as a critical drug target for the development of pharmaceuticals against a number of cancers (2).

The proteasome 20S core particle (CP) is a protein complex that catalyzes peptide bond hydrolysis of substrate proteins and is found in the cytoplasm and nucleus of eukaryotic cells as well as in Archaeobacteria (1). The CP is constructed as a hollow cavity that sequesters catalytic sites involved in protein degradation, with access of substrates to these sites occurring through gated pores, 13 Å in diameter, that are located at opposing ends of the cavity. In the simplest type of CP, found in archaea, each heptameric ring is constructed from one of two structurally homologous proteins, α or β , arranged as $\alpha_7\beta_7\beta_7\alpha_7$, with gated pores and catalytic sites formed by α - and β -subunits, respectively (3). Eukaryotic CPs are more complex versions, whereby each ring is composed of seven distinct α - and β -subunits (4).

Important aspects of the mechanism of the peptide bond hydrolysis reaction are conserved in CPs from all species. Key chemical groups include the hydroxyl on the Thr1 side chain of the β -subunit (β Thr1) that acts as a nucleophile in the peptide bond hydrolysis reaction, the β Thr1 α -amino group that functions as a proton acceptor, and several ionizable groups located on the side

chains of nearby residues that are important for maintaining the structural integrity and reactivity of the catalytic Thr. In addition, there are at least two identified substrate specificity pockets, S1 and S3, that bind amino acid side chains of the substrate at sites N terminal to the point of cleavage; the composition, and therefore the substrate preference, of the pockets varies among β -subunits in a eukaryotic CP, but their locations are conserved with respect to the other groups that directly participate in catalysis (2–4).

The similarities extend beyond the catalytic mechanism to include the processes by which substrates are granted access to catalytic sites. For example, the N-terminal tails of the α -subunits can adopt specific conformations that either expose or occlude the passageway through which substrates enter the barrel of the CP (5). Although the conformations of these tails vary among CPs from different species, the residues proximal to the gate that stabilize the open and closed conformations are highly conserved and gate opening can be induced on interaction of these residues with activator complexes (6–8), otherwise known as regulatory particles (RPs). Such activators include (i) the heptameric 11S RPs (otherwise known as PA28/PA26/REG) that stimulate hydrolysis of peptides and are implicated in the immune response, (ii) the 19S RP that recognizes substrates with ubiquitin tags and degrades them in an ATP-dependent manner, and (iii) Blm10/PA200, whose binding stimulates the degradation of peptides, although the function of this 250-kDa single polypeptide chain is unclear. Detailed structural studies of all three classes of RP rigorously establish that all known activators share a common binding mode, in which the C termini of subunits comprising the RP dock into a pocket that is located at the interface between α -subunits of the CP α -ring. Many of the interactions that form between the CP and RP are independent of the sequence composition of the activator C terminus, and include several hydrogen bonds between the main chains of the RP and α -subunits, as well as a hydrogen bond between the carboxylate group of the C-terminal amino acid of the RP and the side chain of a highly conserved residue in the α -subunit, α K66 (9). The conservation of the binding mechanism is further established by the fact that each 11S RP is able to activate CPs from many different species; for example, the 11S from *Trypanosoma brucei* activates rat and yeast proteasomes, as well as the 20S archaeal CP, even though archaea do not contain 11S RPs (6).

The degradation activities of the 20S CP are used for multiple biological functions for which the activity and sequence preference of the peptide bond hydrolysis reaction is tuned. As mentioned above, it has been clearly established that RPs both select substrates

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to be degraded and grant access of these substrates to catalytic sites by opening gates on binding to the CP (5, 9). However, it is also possible that RP binding could alter proteolysis by allosterically inducing a change to the catalytic sites (10). This possibility has not yet been supported by structural studies of the proteasome. No differences in the structures of the β -subunit are found in X-ray structures of complexes of the CP with the 11S (6, 11), Blm10 (12), or the C-termini of the PAN regulatory particle, in comparison to isolated CPs (13). However, biochemical studies show that proteolysis can be allosterically modulated (10, 14–16) and that modification of active sites leads to gate opening (17, 18), to changes in binding affinities for RPs, and to increases in the stability of CP–RP complexes (19, 20).

Here, we have used solution NMR spectroscopy to provide structural evidence of an allosteric pathway linking the binding sites of RP C termini with the active sites in the *Thermoplasma acidophilum* 20S CP through contiguous segments of structure. This pathway communicates RP binding to the active site, as we demonstrate in the case of binding of the 11S RP, or on mutation of α L81, a key residue that makes contact with the C termini of all known RPs (7). Furthermore, modifications to the CP active sites are communicated to the RP binding site and gate via this same pathway acting “in reverse.” These data demonstrate why high-resolution structural models that consist of a single conformation of the CP cannot rationalize biochemical data. Moreover, the discovery and characterization of an allosteric pathway in the proteasome offer unique opportunities for the design of novel proteasome drugs.

Results and Discussion

NMR Chemical Shift Perturbations of CP Methyl Groups upon 11S RP Binding or Mutation. Because of the large size of the 20S CP (670 kDa) and of the CP–11S complex (1 MDa) we have used the *T. acidophilum* proteasome in all our studies. As described previously, this archaeal version presents a number of important advantages for NMR studies. First, because only single types of α - and β -subunits comprise the CP, rather than the seven different α , β -subunits that are found in eukaryotic CPs, there is both a significant simplification to NMR spectra and an important gain in sensitivity. Second, it derives from a thermophilic species and can withstand temperatures as high as 70 °C; this is conducive to NMR analysis because peaks in spectra, in general, become more intense with increasing temperature due to faster rates of macromolecular tumbling. We have exploited these properties in previous studies of the *T. acidophilum* CP, focusing almost exclusively on α -subunits of either the single ring (α_7), double ring ($\alpha_7\alpha_7$), or full proteasome ($\alpha_7\beta_7\beta_7\alpha_7$) using Ile (δ 1), Leu, and Val methyl groups as probes ($^{13}\text{CH}_3$ -ILV) in an otherwise highly deuterated environment (16, 21, 22) and a methyl-TROSY effect that significantly improves both spectral resolution and sensitivity (23). Near-complete assignments were obtained for α -subunit methyl groups in the context of [^2H , $^{13}\text{CH}_3$ -ILV]- α -, [^2H]- β -labeled CP samples (21), referred to in what follows as ILV- α , β CPs.

These assignments have been verified and extended here (89 of the 95 methyls assigned). These were supplemented by assignments for 72 of the 73 I,L,V methyls in the β -subunits, such that a large number of structural probes, dispersed throughout the complex, were available for analysis (Fig. 1A and *SI Appendix*, Fig. S1). Binding of the 11S activator (PA26) to the α , ILV- β CP ([^2H]- α , [^2H , $^{13}\text{CH}_3$ -ILV]- β CP; $K_D = 10 \mu\text{M}$) (21) produced significant chemical shift changes in 20 of the 38 peaks that were well resolved in the complex (Fig. 1B–E). These changes do not result from direct contact with the activator because binding is to the α -ring; rather, they arise from conformational changes that are propagated from the α -rings to the β -rings.

To determine whether or not the changes in the β -subunit are specific to 11S binding or occur more generally on perturbing the binding interface, CPs were made with mutations at α L81, a highly

conserved residue whose side chain contributes to a hydrophobic surface patch that binds C termini of all known regulators. Studies by other groups have revealed the importance of α L81 in binding of the PAN RP that induces gate opening of the CP and promotes substrate hydrolysis (13). Remarkably, the same methyl groups shift in the same direction and with magnitudes that follow the relation 20S CP α L81A < 20S CP α L81V < 20S CP-11S to produce linear ^{13}C chemical shift vs. ^1H chemical shift profiles (Fig. 1C–E and *SI Appendix*, Fig. S2). We have repeated these experiments, focusing on changes to the chemical shifts of methyl probes in the α -subunits of the CP, labeled as ILV- α , β CP. Because changes in chemical shifts may reflect the direct effect of the mutation rather than the long-range perturbations of interest, we have limited our analysis to resolved methyl groups further than 10 Å from the site of mutation (70 methyl groups; *SI Appendix*, Fig. S3). Again, linear chemical shift profiles are obtained, with the exception of a few residues localized to secondary structural elements containing the mutation. The simplest interpretation of these data is that the α , β -subunits of the CP interchange rapidly between two or more conformations, with exchange that is fast on the NMR chemical shift time scale so as to generate a single average NMR resonance for each probe, as has been observed in NMR studies of other protein systems (24–29). From the largest chemical shift difference (0.5 ppm for ^{13}C , static magnetic field of 18.8 T), an exchange rate significantly in excess of 700 s^{-1} is estimated. To summarize, the effect of the α L81 mutations and 11S binding is to shift the position of an equilibrium involving a set of distinct conformers. This shift and the concomitant changes induced in the α , β -subunits are not specific to 11S binding but occur more generally on perturbing the RP binding pocket.

Our results show that perturbations introduced via binding of the 11S RP or mutations to α L81 extend from the α -ring to the β -subunits of the CP. To establish whether changes to the active sites could lead to global conformational changes, particularly extending to the α -rings, a number of modifications to catalytic residue β T1 have been made. These include (i) β T1A with a six-residue “GSGGGG” prosegment, β T1A-pro; (ii) β T1A with a two-residue “GM” prosegment, β T1A-GM; and (iii) β T1C (Fig. 2A). It has been shown that specific modifications of the active site can result in gate opening (17) or altered binding affinity of the CP for activators (19, 20), at least in the case of the *Saccharomyces cerevisiae* CP. We have measured the extent of gate opening for these three active site variants in the *T. acidophilum* CP by quantifying the relative intensities of correlations arising from the α M-1 and α M1 residues that are part of the α -subunit N terminus that functions as a gate (Fig. 2B). As illustrated previously, separate peaks are observed in ^{13}C , ^1H correlation spectra for Met gating residues corresponding to discrete conformations of N termini either inside or outside the lumen of the barrel (22) (Fig. 2C). For the WT CP, 25.0 \pm 2.0% of the termini populate the “in” states, similar to β T1C, whereas for β T1A-pro and β T1A-GM, the “in” conformation decreases to 16.5 \pm 1.0% and 15.0 \pm 2.0%, respectively (Fig. 2D). These results suggest that gate opening does not result from the length or amino acid identity of the prosegment or from alteration of the amino acid side chain of β T1 but from modification of the α -amino group via attachment of the prosegment. Moreover, our results parallel observations from atomic force microscopy (AFM) studies of *S. cerevisiae* CPs, whereby binding of specific inhibitors or acetylation of the N terminus was shown to result in gate opening (17), further establishing the close similarities between CPs from different species.

For the 74 and 59 methyl correlations that could be quantified in spectra of the α - and β -subunits, respectively, of β T1A-pro and β T1C CPs, 33 and 42 shift in position compared with the WT CP by more than 5 Hz. Although it is not possible to determine the basis of differential gate opening of these active site perturbations (*SI Appendix*, Fig. S4), the effect on the CP conformation is related to that observed for the α L81 mutant and 11S-bound CPs.

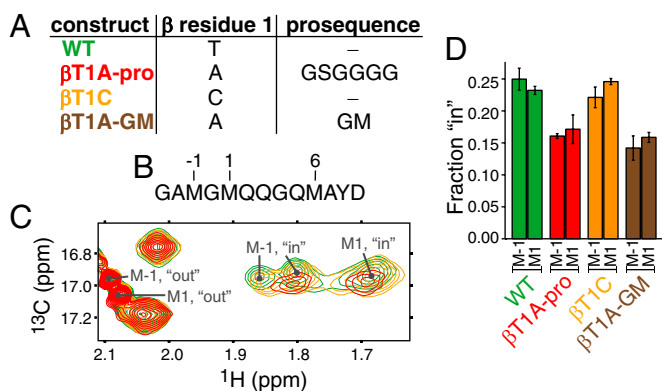


Fig. 2. Specific active site modifications lead to changes in the populations of the “in” and “out” states of the CP gate. (A) Table listing the constructs used to quantify the gate position shows both the identity of the amino acid residue at β 1 that catalyzes the peptide bond hydrolysis reaction in WT CPs and the sequence of the prosegment. (B) Sequence of the gating residues that comprise the N termini of the α -subunits. The methionine residues that are probed by NMR are numbered. (C) Representative spectra show cross-peaks derived from methyl groups of Met residues that are part of the gate of the α -subunit; these data have been used to characterize the effect of active site modifications on the extent of gate opening. α M1 and α M-1 gate residues in B each give rise to multiple peaks. These include (i) “in” peaks that derive from the subset of α N termini occupying the space enclosed by the CP (i.e., the lumen of the CP), thereby occluding substrate entry, and (ii) “out” peaks that result from the gate conformation that places the α N-terminal residues near the outside surface of the CP, allowing substrates to enter (22). (D) Fraction of gating termini that are either outside (“out”) or inside (“in”) the lumen of the CP was quantitated for the CPs indicated in A from the relative intensities of $^{13}\text{C}_3$ peaks of Met residues at positions -1 and 1 of the N termini of α -subunits, using methods described previously (22).

α L81V (β -subunit shifts) or β T1A-pro (α -subunit shifts) perturbations. It is clear that the “red” methyl groups generate a path, whereas the “black” methyls line the outside of the CP or are localized to regions of structure that are not in direct contact with the path. Notably, β V120 γ 1, located in the S3 binding pocket of the active site, shows significant chemical shift changes as a

function of perturbation (Fig. 4D), potentially leading to a modulation of the products of substrate cleavage. We show below that this is indeed the case.

Notably, all residues that shift are located near interfaces between CP subunits. As an illustrative example, large shifts are noted for methyl groups of α H1, located between β H1 and β H2 of adjacent β -subunits, or β H1, located between α H1 and α H2 that reside on neighboring α -subunits (Fig. 4A and B). These methyl groups do not form contacts with each other because, in general, only a few are present on each of the affected structural elements. Rather, as indicated above, they delineate the regions of contiguous structure that are affected by binding or mutations. Focusing on all methyl groups within the β -subunit, an approximate inverse correlation is observed between the shift difference of a given methyl in WT and α L81V CP samples and the distance to its nearest point of contact within the α -ring (Fig. 5B). All residues that shift are located within ~ 12 Å of this interface. Each β -subunit contacts two α -subunits and two β -subunits in the same ring, as well as two β -subunits in the adjacent ring (Fig. 5A). Although most residues that undergo the strongest perturbations are located at the α/β -subunit interface, some localize to the β -intra-ring interface, as shown in Fig. 5C. This includes β V120 in the S3 substrate specificity pocket that is formed from adjacent β -subunits (Fig. 4D). Fig. 5D–F shows the corresponding plots for residues in the α -ring, focusing on shift differences between WT and β T1A-pro. Again, the majority of the changes occur at the α/β interface, with methyl groups >12 Å from an interface showing no changes in chemical shifts. It is thus clear that the interfaces between subunits are fundamental to the conformational changes that occur in the CP. These changes are likely to be subtle. A recent X-ray structure of the *T. acidophilum* CP showed that binding of the C terminus of the PAN RP resulted in only a 4° rotation of the α -subunits about the CP symmetry axis (31).

To explore the conformational changes in more detail, we have measured methyl ^1H - ^{13}C residual dipolar couplings (RDCs) (32, 33) on samples of β T1A-pro and α L81V CPs. It is expected that the differences in structure will be largest for this pair of CPs because their corresponding methyl chemical shifts lie at opposite ends of ^{13}C vs. ^1H chemical shift profiles, as illustrated in Fig. 3. RDCs can be very sensitive reporters of structure, and in the case of an axially symmetrical complex, such as the CP, they provide

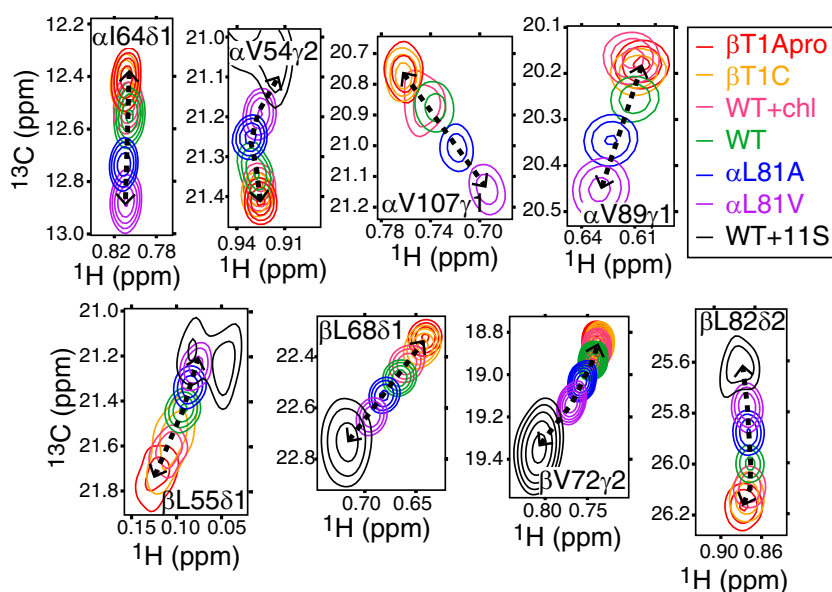


Fig. 3. The CP is in rapid exchange between multiple conformers. Changes in chemical shifts of selected methyl group probes in α - and β -subunits as a function of binding 11S RP, a small molecule inhibitor (chloroquine), or mutations are color-coded as indicated. Dashed arrows are intended to guide the eye.

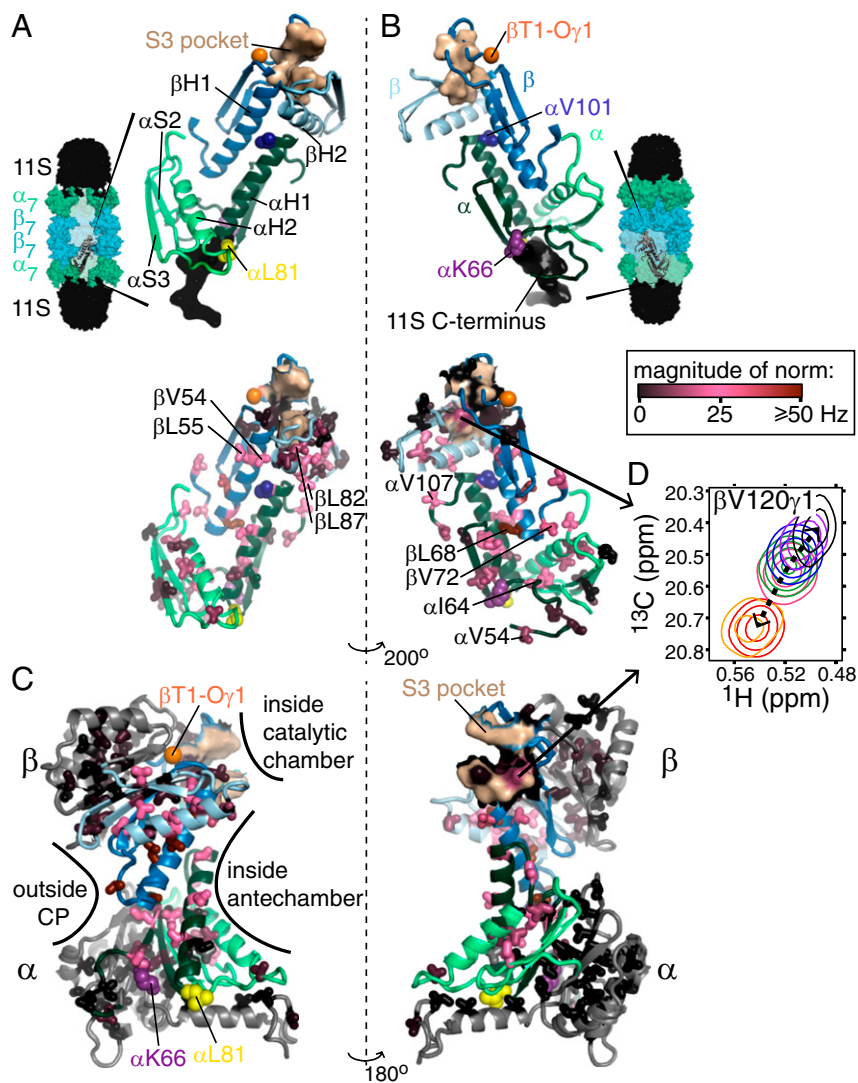


Fig. 4. Contiguous structural region connecting adjacent pairs of α - and β -subunits forms an allosteric network. (A and B) Significant chemical shift changes are localized only to structural elements connecting RP binding with CP active sites. Portions of the α - and β -subunits exhibiting the largest chemical shift changes (*Upper*), derived from pairs of adjacent α - (light and dark green) and β - (light and dark blue) subunits, are shown, with α H1 located at the interface between β H1 and β H2 from adjacent subunits and vice versa. Also shown are these same structural elements, along with the side chains of methyl-containing amino acids that were probed in the experiments (*Lower*), with side chains colored according to the magnitude of the chemical shift perturbations observed for α L81V CPs (β -subunit) and β T1A-pro CPs (α -subunit). Key residues/regions are highlighted, including conserved residues α K66 and α L81, which play critical roles in RP binding; the S3 substrate binding pocket that is modified on RP binding and α L81 mutations; and α V101 and β L82, which make up the chloroquine binding region. Views are from the inside (A) and outside (B) of the CP, as indicated schematically by the CP structures shown that are bound to the 11S RP (black). (C) Single β - and α -subunits (*Upper* and *Lower*, respectively) are shown in two orientations that differ by a 180° rotation, and the side chains of amino acids are colored according to the magnitude of the chemical shift perturbations, analogous to B. The structural elements colored in light/dark green and light/dark blue are the same as those depicted in A and B; however, in that case, they are shown in the context of two adjacent α -subunits and two adjacent β -subunits. (D) Chemical shift perturbations observed for β V120, which is part of the S3 substrate binding pocket, with coloring of peaks as in Fig. 3.

a direct measure of the orientation of each methyl $\text{H}_3\text{C}_{\text{methyl}}\text{-C}$ bond vector with respect to the symmetry axis. RDC data from β T1A-pro and α L81V CPs are in excellent agreement ($R^2 > 0.95$), with a pairwise rmsd of ~ 2.5 Hz, consistent with, at most, very small changes in orientations of methyl axis bond vectors between the different sets of conformers that are sampled by a CP (*SI Appendix, Fig. S6*). The small differences are in keeping with the observed changes in chemical shifts, which are also relatively small at 0.051 and 0.0063 ppm in ^{13}C and ^1H on average, respectively, with maximal changes of 0.54 and 0.04 ppm.

Modulation of CP Cleavage Products by Mutation or 11S Binding.

Because chemical shift changes extend into the S3 active site pocket (Fig. 4D), proteolysis assays were performed to determine

whether this ultimately affects the cleavage products produced. WT, α L81A, α L81V, or 11S-bound 20S CPs were incubated in the presence of a large molar excess of α -synuclein, an intrinsically disordered substrate. Reaction times were kept sufficiently short, with less than 10% of the substrate consumed (*SI Appendix, Fig. S7A and B*), so that the major degradation products reflect initial cleavage of intact α -synuclein rather than further proteolysis of products that reenter the CP after initially exiting (34, 35). Therefore, both the identities and the relative amounts of the different degradation products are characteristic of the reaction over a range of incubation times for which a small fraction of the total substrate is degraded. Reactions were quenched on addition of 0.1% TFA and 5.7 M guanidine-HCl, and peptide cleavage products were separated using reverse phase HPLC (Fig. 6 and

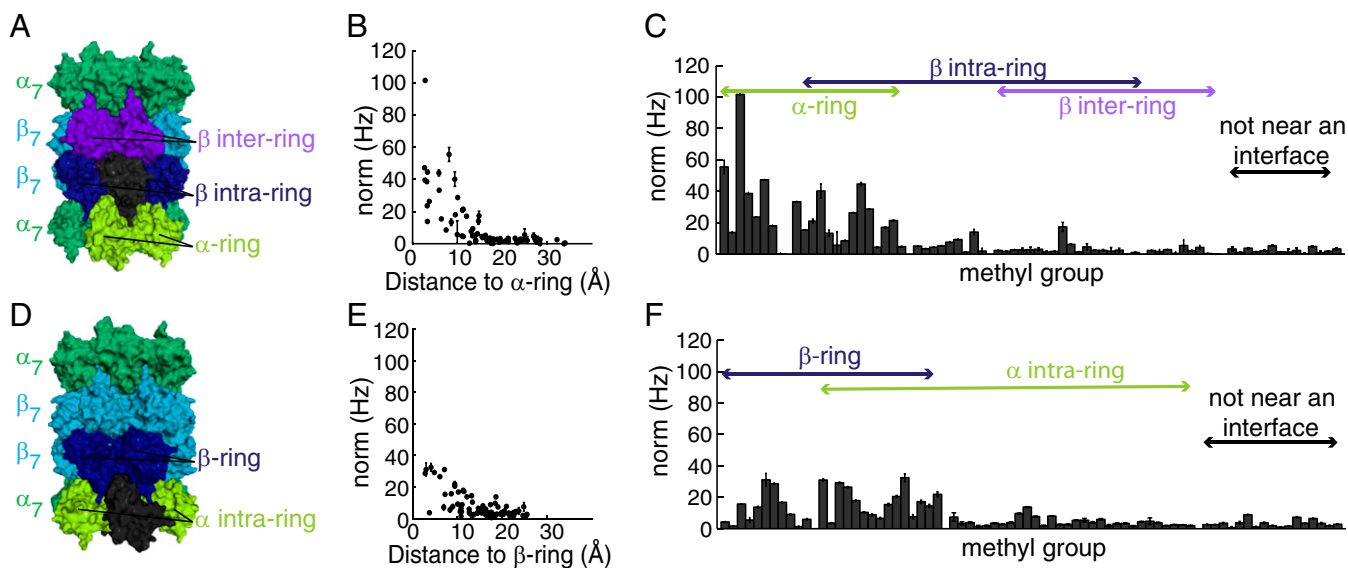


Fig. 5. Interchange between CP conformations involves subunit interfaces. (A) Each β -subunit (gray) is adjacent to a pair of α -subunits (lime) and pairs of β -intra-ring (dark blue) and β -inter-ring (purple) subunits. (B) β -Subunit methyl groups with the largest chemical shift differences (norm) between WT and α L81V CPs are within 12 Å of the α -ring. (C) β -Subunit chemical shift differences (WT vs. α L81V CPs) are largest for methyls at α/β interfaces, with some differences also at intra- β/β interfaces (methyl groups >12 Å from an interface are defined as “not near an interface”). (D–F) As above, but for the α -subunit, with norms computed as chemical shift differences of corresponding α -subunit methyl groups on WT and β T1A-pro CPs.

SI Appendix, Fig. S7). Different CP variants produce HPLC profiles for which the dominant peaks exhibit the same retention times (Fig. 6A), indicating a common set of cleavage products for all CP types. However, the relative amount of each product is altered and unique to each CP variant (Fig. 6A and B and *SI Appendix, Fig. S7*), suggesting that there are changes in propensities of the different substrate sites that are cleaved. To establish that the different cleavage patterns indeed reflect a change in CP preference for each cleavage site rather than differences in mean residence lifetimes for substrate in each of the CPs, we have measured the relative populations of the “in” vs. “out” states of the amino terminal gating residues, as described above and previously (22). Very similar relative populations are obtained for α L81A, α L81V, and WT CPs, consistent with very similar substrate lifetimes (*SI Appendix, Fig. S7*). Taken together, this indicates that the diverse patterns observed reflect differences in the proteasome hydrolysis reaction involving modifications to substrate specificity pockets, as seen by chemical shift changes for β V120Y1 (Fig. 4D), and consistent with shifts in the relative populations of exchanging conformers associated with each “type” of CP assayed. The complexity and multistep nature of substrate hydrolysis do lead, however, to a nontrivial relationship between amounts of cleavage products and relative populations of CP conformers that cannot be elucidated presently.

Intriguingly, the effect on substrate hydrolysis induced by binding of the *T. brucei* 11S RP to the archaeal CP parallels that which has been observed for related 11S–CP complexes, where the 11S and CP were derived from a number of eukaryotic organisms and characterized in both cognate and noncognate complexes (10, 36–39). In the case of eukaryotic CPs, the changes in substrate proteolysis on 11S binding play an important functional role because they result in enhanced production of peptides relevant to the immune response (37–39). Specifically, peptides for which the C termini are generated by proteasome cleavage events are displayed by the MHC class I complex (40). The C-terminal position has an enriched composition of hydrophobic and positively charged amino acids (41), and this coincides with the observed cleavage preferences of the β -subunits of eukaryotic CPs (40). The CP hydrolysis reaction is tuned to generate these

antigenic peptides in a number of ways, one of which is through binding to the 11S RP (40, 41). Interestingly, it has been shown that 11S binding alters not only the proteolysis products produced by CPs from vertebrates that have an immune system but the products that are generated by noncognate complexes involving yeast or insect CPs for which an immune system is absent (38). It is of interest, therefore, to speculate about the possibility of a similar distribution of eukaryotic CP conformers to that observed for the archaeal *T. acidophilum* CP, with 11S binding shifting the relative populations of states here as well, leading to a modulation of

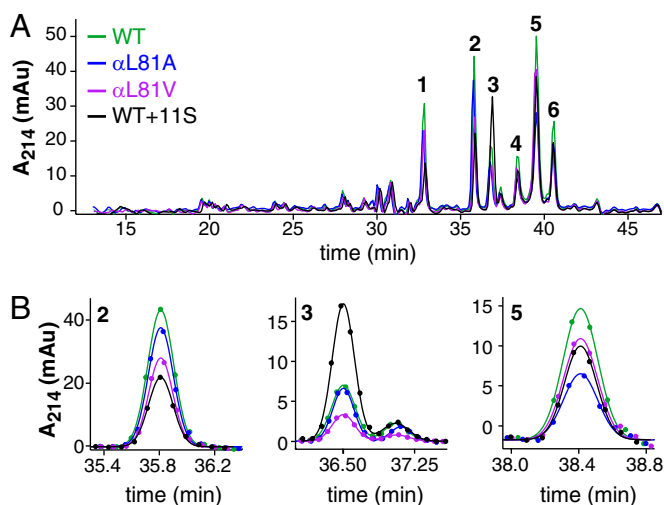


Fig. 6. Relative populations of CP conformations modulate the distribution of cleavage products. (A) Reverse phase HPLC trace of cleavage products generated with different CP variants (0.3 μ M WT, α L81A, α L81V, and 11S-bound) incubated with 300 μ M α -synuclein for 30 min at 50 °C. (B) Comparison of reverse phase HPLC profiles of α -synuclein degradation products shows that similar products are produced with the CP variants but that the relative amounts of each differ. Expansions of traces are shown for peaks 2, 3, and 5. mAu, milli Absorbance unit (UV-visible spectroscopy).

activity and changes in cleavage products that are used in the immune response.

Chloroquine Binds Near the α,β Interface and Modulates Function Through the Allosteric Network. The success of bortezomib, a potent proteasome inhibitor that has been used in the clinic for the treatment of multiple myeloma, has encouraged the design of further novel anticancer drugs that target the CP. Most of these pharmaceuticals, including bortezomib, are based on modification of function through binding to proteasome active sites (2, 42). We have previously described studies of the antimalaria drug chloroquine, which inhibits proteasome function in eukaryotic cell extracts and in preparations of purified 20S CP from *T. acidophilum* (16). Notably, biochemical data have established that chloroquine inhibits CP activity through binding to regions distal from proteolytic active sites (16), suggesting that similar compounds (albeit with higher affinity) could be used synergistically with bortezomib (42, 43).

Previous NMR characterization of chloroquine (Fig. 7*A*, *Inset*) binding to ILV- α , ILV- β CPs ($[U\text{-}^2\text{H}, ^{13}\text{C}_3\text{-ILV}]\text{-}\alpha$, $[U\text{-}^2\text{H}, ^{13}\text{C}_3\text{-ILV}]\text{-}\beta$ -labeled CPs) showed large changes to the chemical shifts of many methyl groups, especially those located at the interface between α - and β -subunits (16). At that time, assignments for peaks in

the β -subunit were not available and a binding site could not be identified. Here, we have used experiments to identify the binding site via magnetization transfer, whereby aromatic groups of chloroquine are selectively saturated, leading to a reduction in the intensity of CP peaks derived from methyl groups located near the site of binding (details are provided in *SI Appendix*). Both methyl groups from αV101 are affected (Fig. 7*A*), indicating proximity to chloroquine, although other methyls from α -subunits or from the β -ring (Fig. 7*A*, *Inset*) are not perturbed. As shown in Fig. 3, chloroquine binding shifts many peaks in the $^{13}\text{C}, ^1\text{H}$ CP spectrum, analogous to what is observed on mutation at position αL81 , modification to the active sites (βT1), or binding of 11S. Notably, however, several peaks from methyl groups that are located near αV101 shift in directions distinct from those observed previously, reflecting the formation of direct interactions with the drug or distortions in the structure of the CP near the binding site. For example, the two methyl groups of αV101 shift in a direction that is unequivocally different from what was observed in other CP samples (i.e., not on a line) and with a magnitude that is 4.8-fold larger than the maximum shift observed in all the other CP variants (Fig. 7*B*). The chemical shift perturbations for the $\beta\text{L55}\delta\text{1}$ and $\beta\text{L82}\delta\text{1}$ methyl groups, showing the next largest effects, are also distinct, but these are much smaller. βL55 and βL82

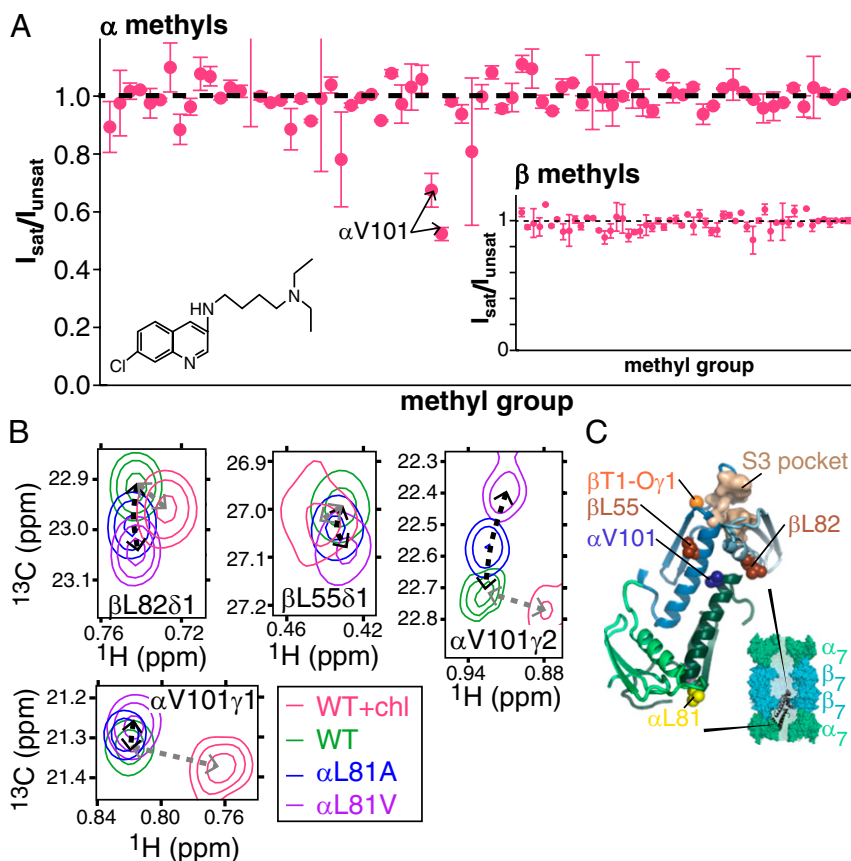


Fig. 7. Location of the chloroquine binding site. (A) Saturation transfer experiments indicate that chloroquine (structure in *Inset*) binds near αV101 . Samples consisted of chloroquine mixed with α , ILV- β or ILV- α , β CPs with equimolar concentrations of chloroquine and CP α/β -subunits. Peak intensities of CP methyl groups were compared in pairs of spectra acquired, with the center of the saturation bandwidth (± 1 ppm) applied either at 7.9 ppm (I_{sat}), which selectively saturates chloroquine aromatic protons, or at -6.5 ppm (I_{unsat}), a site approximately the same distance from the methyl groups as the aromatics. To minimize artifacts resulting from a small amount of saturation in the control (unsat) experiment or magnetization transfer that is unrelated to binding (49, 50), $I_{\text{sat}}/I_{\text{unsat}}$ has been renormalized by the intensity ratio obtained by measurement in an analogously prepared sample that lacked chloroquine. Renormalized values are plotted along the y axes. (B) CP methyl group chemical shifts indicate that the chloroquine binding site is located near αV101 . Notably, several peaks shift in a manner distinct from the perturbations that occur in methyl groups along the allosteric pathway (Fig. 3), reflecting chloroquine binding. (C) Locations of residues whose methyl chemical shift changes reflect binding are shown, where the CP is depicted in a manner analogous to that in Fig. 4*A*.

are two of the three methyl-containing residues in the β -subunit that are located closest to α V101 (methyl-methyl distances of 9.6 and 9.7 Å, respectively). The chemical shift perturbations are thus consistent with saturation transfer measurements, indicating that chloroquine binds proximally to α V101 (Fig. 7C), 23 Å removed from the nearest active site. Inhibition of CP function by chloroquine is thus allosteric, consistent with previous studies by our group showing that chloroquine inhibition is noncompetitive and that both chloroquine and MG132, a competitive inhibitor that binds to the CP active sites, can bind simultaneously (16).

After omitting methyl groups proximal to the site of chloroquine binding, the largest shifts occur for residues located at the interface between the α - and β -subunits (16) (Fig. 8 and *SI Appendix*, Fig. S8), as has been observed for other CP variants as well (Fig. 5). In the majority of cases, those peaks that shift do so along the same curves defined by methyl groups from the α L81 mutant, the β -active site variant, and 11S-bound CP forms (Fig. 3 and *SI Appendix*, Fig. S8A). Moreover, the directions of the shifts are consistent with those observed for β -active site variants (*SI Appendix*, Fig. S8B–G), which is expected because both chloroquine binding and β T1 modification result in the inactivation of the CP. Thus, chloroquine shifts the equilibrium between exchanging CP states in a direction that leads to inhibition.

Concluding Remarks. Although high-resolution structural analyses of the CP have indicated a single conformation (6), our results show that in solution, a distribution of structurally distinct states is present. These states link protein hydrolysis by and RP binding to the 20S CP via an allosteric pathway comprising contiguous elements of structure-connecting regions that are 80 Å apart. Manipulation of the pathway through mutation or binding of the 11S regulator shifts the relative populations of states that rapidly interconvert, leading to changes in substrate hydrolysis. The allosteric pathway described here for the *T. acidophilum* CP may well be operative in more complex CPs as well, because CPs from all species share many common features. For example, (i) both the mechanism of proteolysis (2) and the regulation of function through binding of RPs (9) are conserved across CPs; (ii) changes in relative amounts of substrate cleavage products that have been quantified here for *T. acidophilum* CP have also been observed for both cognate and noncognate 11S–CP complexes, particularly for CPs derived from eukaryotes (10, 36–39); (iii) similar changes to the in and out gate conformations have been

observed in both archaeal and *S. cerevisiae* CPs via modifications to β T1; and (iv) both *T. acidophilum* and eukaryotic CPs have been shown to be inhibited by the allosteric inhibitor chloroquine. The identification of such a path offers a unique avenue for the design of new classes of inhibitors that may ultimately play an important role in the treatment of proteasome-related diseases (42, 44).

Materials and Methods

Constructs of the α - and β -subunits were expressed, purified, and assembled into CPs as described previously (45). Samples were produced with appropriate isotopic labeling for methyl TROSY NMR experiments by expressing the CP subunit of interest in M9 minimal media, 99% D₂O (99 atom % D) with [¹²C, ²H] glucose as the sole carbon source. To introduce methyl labels, the appropriate precursors were added 1 h before induction [60 mg/L 2-keto-3,3-D₂-4-¹³C-butyrate for Ile, 80 mg/L 2-keto-3-methyl-D₃-3-D₁-4-¹³C-butyrate for Leu/Val, and/or 100 mg/L methyl-¹³C L-Met for Met, as described previously (22, 46)].

All spectra were acquired on a Varian Inova spectrometer (18.8 T) equipped with a room temperature pulse field gradient triple resonance probe. Assignments of α -subunit ILV-methyl groups in the CP were published previously (21), and those for the β -subunit were determined by the process outlined in *SI Appendix*, Fig. S1. CPs were buffer-exchanged into 25 mM potassium phosphate (pH 6.7), 50 mM sodium chloride, 0.03% azide, 1 mM EDTA, and 99.9% D₂O using a concentrator (30- or 50-kDa molecular mass cutoff). Chemical shifts are reported for methyl groups in the α -subunit from measurements on samples that had the following concentrations (expressed in terms of the α -subunit): 770 μ M for α L81V CP, 790 μ M for α L81A CP, 1,000 μ M for WT CP, 500 μ M for β T1A-pro CP, and 1,500 μ M for β T1C CP. In the case of samples used to measure β -methyl group chemical shifts, the concentrations of the β -subunit were as follows: 280 μ M for α L81V CP, 330 μ M for α L81A CP, 550 μ M for WT CP, 770 μ M for β T1A-pro CP, and 750 μ M for β T1C CP. For measurements of 11S binding, samples consisted of 300 μ M CP and 1.2 mM 11S (conditions in which binding to the CP is saturated) in 25 mM Hepes (pH 8.5), 0.03% azide, 1 mM EDTA, and 99.9% D₂O at 50 °C. The ¹³C,¹H correlation spectra were recorded using a standard heteronuclear multiple quantum coherence (HMQC) pulse scheme that takes advantage of a methyl-TROSY effect (23). Typically, datasets for measuring Ile, Leu, and Val correlations were obtained with 24-ms and 64-ms acquisition times in t_1 and t_2 , respectively, and with a relaxation delay of 1.5 s. For spectra in which gate populations were quantitated (Fig. 2C) by measuring volumes of α -subunit Met correlations, maximum t_1 and t_2 acquisition times were both set to 80 ms and a relaxation delay of 3 s was used. NMR spectra were processed using NMRPipe (47), and chemical shifts for peak centers were measured using the program SPARKY (48). In the case in which spectra of the CP-11S complex (50 °C) were compared with datasets from all other CPs (70 °C), control experiments were conducted to ensure that chemical shift perturbations were not significantly altered by differences in buffer composition or temperature.

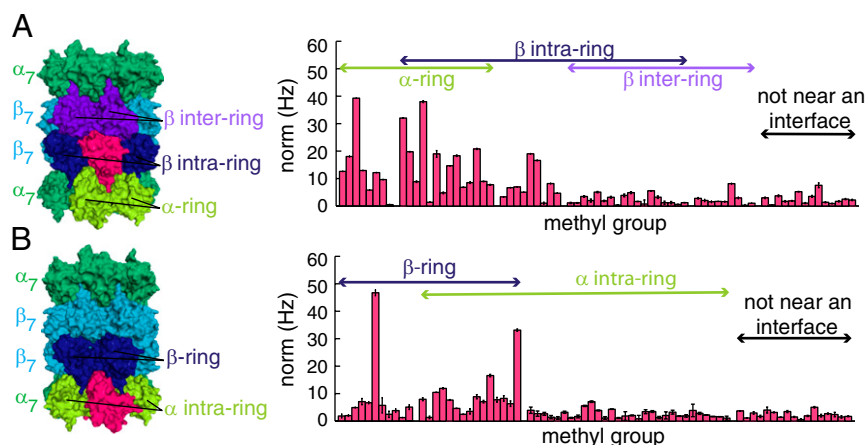


Fig. 8. Binding of the allosteric inhibitor chloroquine to the CP involves subunit interfaces. Histograms with bar heights corresponding to “norm” values but grouped according to the corresponding methyl group’s proximity to subunit interfaces [β -methyl groups (A) and α -methyl groups (B), respectively] show that the largest chemical shift changes occur for methyl groups located near the interfaces between CP subunits. A methyl group is considered to be proximal to an interface if the distance to its closest point of contact on an adjacent subunit is ≤ 12 Å. Each of the interfaces between subunits is defined with respect to the subunit colored in pink.

For each NMR sample, data were acquired as two to six separate successively measured HMQC spectra. Chemical shifts were extracted from each spectrum, and the values reported reflect the mean measured for each peak across all spectra, with error bars corresponding to the SD. All chemical shifts were referenced against 2,2-dimethyl-2-silapentane-5-sulphonic acid. Further experimental details are provided in *SI Appendix*.

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