Differential global structural changes in the core particle of yeast and mouse proteasome induced by ligand binding

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Two clusters of configurations of the main proteolytic subunit $\beta 5$ were identified by principal component analysis of crystal structures of the yeast proteasome core particle (yCP). The apo-cluster encompasses unliganded species and complexes with nonpeptidic ligands, and the pep-cluster comprises complexes with peptidic ligands. The murine constitutive CP structures conform to the yeast system, with the apo-form settled in the apo-cluster and the PR-957 (a peptidic ligand) complex in the pep-cluster. In striking contrast, the murine immune CP classifies into the pep-cluster in both the apo and the PR-957-liganded species. The two clusters differ essentially by multiple small structural changes and a domain motion enabling enclosure of the peptidic ligand and formation of specific hydrogen bonds in the pep-cluster. The immune CP species is in optimal peptide binding configuration also in its apo form. This favors productive ligand binding and may help to explain the generally increased functional activity of the immunoproteasome. Molecular dynamics simulations of the representative murine species are consistent with the experimentally observed configurations. A comparison of all 28 subunits of the unliganded species with the peptidic liganded forms demonstrates a greatly enhanced plasticity of $\beta 5$ and suggests specific signaling pathways to other subunits.

20S proteasome | PCA analysis | allosteric regulation

Among the many factors involved in protein degradation through the ubiquitin-proteasome pathway, the core particle (CP) 20S proteasome plays the key role of the protease component. With the regulatory particle (RP), it forms a complex that selectively degrades ubiquitin-protein conjugates (1, 2). The CP in eukaryotes is a multisubunit complex composed of four stacked heptameric rings: two identical outer rings formed by seven different α subunits and two identical inner rings formed by seven different β subunits. The $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ organization defines a cylindrical structure (3). The α -rings control substrate entry into the lumen of the particle, where it is processed at the peptidolytic active centers, which are located at the inner walls of the β rings, specifically at subunits $\beta 1$, $\beta 2$, and $\beta 5$. These active subunits are characterized by an N-terminal Thr residue. The other four β subunits have unprocessed N-terminal propeptides and are enzymatically inactive.

All three active subunits share a common peptide hydrolyzing mechanism with two main steps (4): (i) the positioning of the substrate peptide in the active site by antiparallel alignment in between segments 47–49 and 21 of the active β subunits and (ii) peptide bond cleavage initiated by a nucleophilic attack of the hydroxyl group of the N-terminal Thr1 on the carbonyl carbon atom of the scissile peptide. Sequence diversity among β subunits endows them with distinctive structural features and different specificity pockets (S1, S2, S3, etc.) where the substrate side chains (P1, P2, P3, etc.) are bound (5). Consequently, the correlation of structural features of the S1 pockets with the distinctive

cleavage products has led to the association of $\beta 1$, $\beta 2$, and $\beta 5$ with caspase-like, trypsin-like, and chymotrypsin-like activities, respectively (6).

The catalytically active subunits are substituted in immune cells of vertebrate organisms by the immune β -subunits $\beta 1i$, $\beta 2i$, and $\beta 5i$ as part of an adaptive immune response. These substitutions cause substantial functional differences between the constitutive (cCP) and immune (iCP) species, reflected in higher yield of peptides that are recognized by the major histocompatibility complex (MHC) class I generated by iCP (7). Additionally, it has been observed that iCP achieves higher degradation rates than cCP, in both in vitro and cellular assays (8–13).

Some sequence variations between the constitutive and immune subunits provide explanations to the observed catalytic differences. Most conspicuously, and first seen in the eukaryotic proteasome crystal structure from yeast (yCP) (3) and confirmed by the murine constitutive and immune CP structures (mcCP and miCP) (14), Arg45 of the $\beta 1$ subunit, located at the base of the S1 pocket, is replaced by leucine in $\beta 1i$, thereby causing a specific change of the electrostatic milieu, in line with the observed low postacidic activity of the iCP (15).

Despite the high sequence similarity between $\beta 5$ subunits of mcCP and miCP including identical active sites, a peptidic α - β -epoxyketone inhibitor, PR-957, showed higher affinity to iCP by one order of magnitude. The structural comparison of cCP

Significance

We analyzed 46 molecular structures of the yeast proteasome core particle (CP) by principal component analysis (PCA) and discovered two distinct configurations of the principal proteolytic subunit $\beta 5$: the apo-cluster encompassing complexes with nonpeptidic ligands and the pep-cluster of complexes with peptidic ligands. Both configurations differ by a small domain motion and numerous slight global changes, thus enabling intersubunit communication. PCA was expanded to the mouse CP and revealed a striking difference between the constitutive CP and the immune CP. The former conforms to the yeast system and executes the structural change seen in yeast, although both immune apo and liganded CP classify into the pep configuration, a possible explanation for the generally higher activity of the immune proteasome.

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Data deposition: The structure has been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4QBQ).

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and iCP in their apo and PR-957 liganded states suggested an explanation. On binding of PR-957, the cCP $\beta 5$ backbone displays significant deformations, whereas the iCP $\beta 5$ backbone remains unchanged. This observation, together with our experience in constructing $\beta 5$ models for virtual screening purposes, prompted us to reinvestigate the vast amount of structural data for yCP by a procedure that facilitates discovery of global changes: principal component analysis (PCA).

We focus our study on the $\beta 5$ subunit, because $\beta 5$ inactivation in yeast renders a lethal phenotype (16) and therefore $\beta 5$ harbors an essential enzymatic activity, and because almost all crystallographically defined complexes are liganded at their $\beta 5$ active site.

Here we present a detailed investigation of the wealth of yeast and mouse proteasome ligand complex structures that led us to embark on structural comparisons beyond the immediate vicinity of the ligands to obtain a view of the global response of the core particle of yeast and mouse proteasome to complex formation. This study (i) is evidence of the structural plasticity of the β , specifically $\beta 5$, subunits; (ii) offers perspectives for the analysis of the structure-function relationship of the CP; and (iii) provides an aid for the design and development of ligands as drugs for this intensively studied target for cancer and autoimmune diseases.

Results

Structural Transition of Subunit $\beta 5$ on Peptidic Ligand Binding. We analyzed 46 $\beta 5$ subunits from yCP crystal structures (y $\beta 5$) reported in the Protein Data Bank (PDB) database (Tables S1 and S2) aiming to identify backbone transitions induced on inhibitor binding by PCA because it eases structure classification (17–19).

The projection of each structure onto the first two PCA eigenvectors revealed two clusters clearly distinguished by the first principal component (PC1) (Fig. 1). Although PC1 captures 78% of the structural variances, the second principal component (PC2) accounts for less than 5% (Fig. 1, *Inset*). The clusters are highly correlated with inhibitor binding. On the positive x axis, structures in either the apo-state or in complex with nonpeptidic inhibitors are found, whereas the projections on the negative side correspond to structures with peptidic inhibitors. In the following, we refer to these clusters as apo- and pep-clusters, respectively. Striking results are obtained by projecting the $\beta 5$

Fig. 1. Projection of the $\beta 5$ structures on the first two eigenvectors of PCA (PC1, PC2). Structures of y $\beta 5$ (x), m $\beta 5c$ ($\square$), m $\beta 5i$ (∇), and b $\beta 5c$ ($\triangle$) are classified into two groups; structures in complexes: (i) peptidic inhibitors (red) and (ii) nonpeptidic inhibitors or apo structures (black). Filled symbols indicate structures in complex with PR-957. The Boc-(Ala) $_3$ -al yCP complex is represented by $\circ$. The arrows indicate the position of apo (1RYP) and Bor-tezomib complexed (2F16) structures. (*Inset*) Eigenvalue spectrum representing the percentage of the total variance captured by the corresponding eigenvector.

structures of the murine (m $\beta 5$) and bovine (20) (b $\beta 5$) species on the eigenvectors of the PCA analysis of the yeast structures. The constitutive subunits, m $\beta 5c$ and b $\beta 5c$, are classified into the apo-cluster in their ligand free forms, whereas the uncomplexed structure of the immune subunit, m $\beta 5i$, colocalizes in the pep-cluster together with the mammalian liganded structures.

These observations led us to investigate the contribution of the amino acid side chains of the peptidic inhibitors to the observed structural perturbation. Thus, we synthesized the peptidic Boc-(Ala) $_3$ -al inhibitor (*SI Methods*) and determined the yCP cocrystal structure with the aim to discern contributions from the main chain and side chains. The small and neutral methyl groups do not fill the subsites and are expected to exert minimal influence. Interestingly, the complex structure clusters with the pep-series (Fig. 1), thus excluding a decisive role of the side chains and underpinning the importance of the main chain on the classifications.

To highlight the domain motion induced by binding of peptidic ligands, structures from each of the two clusters were overlaid considering residues 1–39 and 125–190 (Fig. 2). The protein segments containing residues T21, G47, and A49 accept and donate, respectively, four hydrogen bonds to peptidic ligands, configuring a short three-stranded antiparallel β -sheet (Fig. 2A). This binding appears to trigger the closure of the pocket relative to the apo-series by shifting the α -helix (H1), comprising residues 49–70, of y $\beta 5$ (Fig. 2B). A similar reorganization is observed for the m $\beta 5c$ on binding of a peptidic inhibitor (Fig. 2C) but is absent in m $\beta 5i$, whereas both apo and liganded structures present a closed conformation to establish the antiparallel β -structure (Fig. 2D). Similar comparison of apo and peptidic liganded bacterial proteasomes, using the mycobacterial CP structures (21), did not indicate molecular rearrangements as seen in yeast.

To support the previous observations on the role of peptidic binding, we performed a series of molecular dynamics (MD) simulations of 20 ns length on a truncated model of the CP (*SI Methods* and Table S3). We simulate m $\beta 5c$ and m $\beta 5i$ under three different starting conditions: (i) apo structures, (ii) structures in complex with a peptidic ligand, and (iii) complexed structures but with the ligand removed. The stability of the open and closed conformers is backed up by the simulations (Fig. 3 and Fig. S1). Notably, projecting the MD trajectories of the apo and the liganded forms onto PC1, computed from yeast coordinates, shows that during the simulated time, the structures remain within their corresponding apo- and pep-cluster regions (Figs. 1 and 3 and Fig. S1). In contrast, deletion of the ligand from m $\beta 5c$ and m $\beta 5i$ impacted the simulation very differently. After 400 ps (Fig. 3) and 2 ns (Fig. S1) in two independent runs, the m $\beta 5c$ shifts from the pep- to the apo-cluster region, remaining there for the rest of the simulation, whereas m $\beta 5i$ stays in the pep-cluster region. Both runs of the MD trajectories sampled regions that deviate quantitatively from those defined by the PCA analysis of the yeast structures (Fig. 1), but the histograms reveal a qualitative agreement and clearly two populations, the apo- and pep-clusters, respectively. We attribute this quantitative deviation to the MD equilibration process of the crystal structures and to the truncated approximation of the CP. Taken together, the PCA analysis (Fig. 1), the structural overlays (Fig. 2), and the MD simulations (Fig. 3 and Fig. S1) document that m $\beta 5c$ follows the pattern seen in yeast, showing a domain closure on peptide binding and hydrogen bond formation. Notably this transition does not occur in m $\beta 5i$, where both apo and pep structures remain in the closed conformation.

Implications of Peptidic Binding on the CP Structure. An identical PCA analysis for all 14 subunits indicated similar, albeit less pronounced, clustering only for $\beta 4$ and $\beta 6$, which are adjacent to $\beta 5$ in the heptameric ring. Nonetheless, to further explore whether the state of the $\beta 5$ active site has an influence on the rest

Fig. 2. Domain motion in subunit $\beta 5$ induced by binding of peptidic ligands. (A) Peptidic binding at the active site of yeast $\beta 5$ (apo in black, liganded in red). (B) Structural superposition of $\gamma\beta 5$ structures: apo (1RYP, black) and complex with a peptidic inhibitor (2F16, Bortezomib, red). (C) Superposition of $m\beta 5c$: apo (3UNE, green) and complex with a peptidic inhibitor (3UNB, PR-957, blue). (D) Superposition of $m\beta 5i$: apo (3UNH, green) and complex with a peptidic inhibitor (3UNF, PR-957, blue). Arrows highlight the domain movement of $\gamma\beta 5$ and $m\beta 5c$. Ligands are not shown in B–D for clarity.

of the CP structure, each of the 13 remaining subunits was analyzed from the $\beta 5$ perspective. For this purpose, the structures were classified into the apo-cluster, the elements of the $\beta 5$ pep-cluster with $\beta 5$ specific ligands, and the elements of the $\beta 5$ pep-cluster with ligands also bound at $\beta 1$ and/or $\beta 2$. Average structures from these three sets were computed, and the differences for each subunit in their $C\alpha$ coordinates were mapped along the polypeptide chain (Fig. 4). In accordance with the PCA data, the binding of a peptidic inhibitor at $\beta 5$ induces significant backbone shifts over the whole subunit. Notably, these are propagated to the neighboring subunits: $\beta 4$ and $\beta 6$, and, to a smaller extent, to $\beta 2$. Interestingly, only minor displacements are induced by peptidic binding at $\beta 1$ and $\beta 2$. In contrast to the clear perturbations observed in β subunits, only small and localized signals are observed in the α subunits. The backbone shift in $\alpha 3$ around residue 220 can be associated with the displacements in the β rings. This structural change is further supported by a hydrogen bond between $\alpha 3N_{221}N\gamma$ and $\beta 2D_{220}O$ and by the observed displacement of the last 20 residues of $\beta 2$ that are in close contact with $\beta 3$ and the shifted region of $\beta 6$. Perturbation pathways explaining the other displacements of the α subunits are not evident. Intriguingly, residues known to be involved in the assembly of the 26S particle (residue 66 in the α subunits) and located in proximity of the gate channel (residue 129) are found within the shifted regions. To assess the effect of peptidic binding from a different perspective, the crystallographic “temperature” B (disorder) factors of the main chain atoms were averaged over the same set of structures as in the $C\alpha$ analysis (Fig. S2). Interestingly, the analysis shows that the variations of the B-factors of the β -subunits are substantially higher than in the α -subunits and follow the trend seen for the structural alterations on ligation (Fig. 4). Taken together, the observed variations of $C\alpha$ positions and B-factors testify to enhanced structural plasticity of the β subunits, specifically $\beta 5$, and mark possible pathways for intersubunit communication.

To gain further insights into possible shift conferment pathways, we analyzed $C\alpha$ differences in $\beta 5$ (Fig. 5A). The shift induced in $\beta 5$ propagates to $\beta 6$ and $\beta 2$ through the segments 30–41 and 204–212, whereas the transfer to $\beta 4$ occurs through the segment 115–144. It is worth mentioning that a single $\beta 5$ subunit interacts with $\beta 4$, in both *cis* and *trans*, i.e., within its heptameric ring and across with the adjacent ring, thus enabling communication

to the *trans* $\beta 5$. Helix H1 of $\beta 5$ neighbors $\alpha 4$ and $\alpha 5$ offers a possible signal pathway to the α rings (Fig. 5B). Interestingly, only minor influences are observed in $\beta 3$, despite its proximity to the ligand binding site of $\beta 5$. Aside from these observations, at least five distal segments that are not in direct contact with the active site depict considerable backbone displacements (Fig. 5A). The role of these five segments was examined for the definition of an apo- or pep-structures by a PCA analysis (Fig. 5C). Residues 42–53 produce just enough signal to discriminate among the clusters, which is expected because most of these amino acids are directly involved in peptidic binding. However, when residues 30–41 are considered, a clearer differentiation is observed. This finding is surprising, because most of them are located at the outer surface of the CP, thus far from the ligand binding site. Progressive inclusion of the other distal segments in the analysis increases the distance between the clusters, thereby demonstrating that the signal of peptidic binding propagates to the back of the subunit and thus describing a possible communication pathway between the inner and outer surfaces of the CP. The importance of these segments for peptidic binding at the active site is supported by backbone shifts observed in $m\beta 5c$ (Fig. 5D).

Fig. 3. Molecular dynamics simulation. Projections of the trajectories of six mouse $\beta 5$ structures on PC1 of PCA of the $\gamma\beta 5$ structures. The simulated system corresponds to structures starting from the apo (apo), liganded [pep(+)] and liganded state after removing the inhibitor [pep(-)]. The solid lines represent the running averages of 50 frames windows. The histograms on the right are computed with the raw trajectory data.

Fig. 4. C_{α} differences of each of the 14 different subunits of yCP. C_{α} differences between average apo-structure and average complex structures with (i) peptidic ligand bound to $\beta 5$ (blue) or (ii) peptidic ligands also in other active subunits (red). The associated error is computed from the SD of the averaged coordinates (shaded regions). Dashed lines highlight residues involved in the peptidic binding (black) and forming the H1 α -helix (red), respectively.

Although similar backbone displacements have been identified in $y\beta 5$ and $m\beta 5c$, the $m\beta 5i$ main chain atoms retain the configuration of the pep-structure.

Discussion

The regulation of an enzyme activity by ligand binding at an allosteric, i.e., distant from the active, center is a frequent phenomenon manifested structurally in changes of quaternary structures as in the founding case of hemoglobin, in large-scale domain rearrangements, disorder-order transitions, and global small scale changes. The underlying mechanisms may be a selection from a preexisting population of configurations (conformational selection) or ligand induced structural shifts (induced fit). An unambiguous distinction between these limiting cases requires measurement of the kinetics of ligand binding. A structural definition may be given by the plethora of tools of structural biology including theoretical and molecular dynamics calculations, with X-ray crystallography being a main source of experimental data. However, the detection of small scale global rearrangements from crystallographic data is challenging when using the common simple visual inspection of superimposed atomic models, which are of limited accuracy. PCA has been introduced and applied to the analysis of conformational ensembles from molecular dynamics simulations and offers also a tool for comparing large sets of experimental structures with the aim of detecting and defining common modes of deformation (22, 23).

We were inspired to apply this method on the proteasome crystallographic data for several reasons. In a large set of structures of yeast CP ligand complexes, no conformational changes were described, whereas functional activity measurements indicated an allosteric interaction between the different active sites (24, 25), which should be reflected in structural changes. However, the interpretations were controversial (26, 27).

Additionally, atomic force microscopy (28) and biochemical assay (29) data suggested a correlation between the status of the active site at the β -subunits and features of the α -subunits in yeast CP. In the same line, NMR measurements of the archaeal 20S proteasome indicated an allosteric communication of the active sites with the α -subunits (30, 31). These observations, together with the small domain movement seen in the crystal structures of murine cCP on ligand binding but not in iCP (14), called for a more detailed analysis.

The reinspection of the multitude of differently liganded yCP crystal structures provides further insights into the mechanism of the 20S proteasome. The presented PCA analysis of the yCP was restricted to main chain atoms and focused essentially on the $\beta 5$ active subunits. Surprisingly, it revealed two conformers, apo- and pep-clusters, unveiling differential specific structural changes on ligand binding and deformations by peptidic ligands which form, in contrast to other types of ligands, a characteristic antiparallel β -sheet with the backbone of residues T21, G47, and A49. We ascribe the wide space drawn out by peptidic ligands in the PCA map compared with other ligands (Fig. 1) to the large contact area in their binding pockets, provoking additional differential distortions (Fig. S3). Peptidic ligand binding at $\beta 5$ is the principal trigger of all observed conformational changes in yCP and cCP. Additional binding at $\beta 1$ and $\beta 2$ has much less influence on those subunits and on $\beta 5$ (Fig. 4 and Fig. S4). Covalent linkage with Thr1 by various reactants of either nonpeptidic or peptidic ligands does not have a noticeable bearing on the main chain configurations as defined by PCA analysis. Notably, mammalian CPs blended into the clusters showing that there is a consistent structural transformation on peptidic ligand binding among eukaryotic CPs. The dominant influence of the main chain ligand protein interaction is revealed by the fact that $y\beta 5$ in complex with Boc-(Ala)₃-al clusters with the pep-series. In line with this finding are the results provided by the MD simulations, which give support for the existence of two $\beta 5$ conformations and highlight the role that peptidic binding has in differentiating these conformations. Although the apo and liganded structures of the $m\beta 5c$ and $m\beta 5i$ are stable during the simulation time, the removal of the peptidic ligand from the $m\beta 5c$ causes a switch from the pep-conformation to the apo-structure within 400 ps and 2 ns, respectively, in two runs. In strong contrast and in agreement with the structural data, removing the peptidic ligand from the $m\beta 5i$ structure has no effect.

The tight packing of the subunits in the CP together with the observed conformational changes opens the possibility of signal propagation from $\beta 5$ to other subunits. Most of the conformational alterations in $\beta 5$ induced by the peptidic binding are distant from Thr1 and located at the surface of the CP (Fig. 5A) and hence are not in contact with other subunits and therefore are unlikely involved in direct signal transduction. However, they are evidence for the transit among conformations. Their relevance for the definition of the apo- and pep-clusters is supported by PCA and the murine $\beta 5$ crystal structures (Fig. 5C). Among the backbone displacements that contact other subunits and may generate a direct signal, two are preeminent: the backbone shift induced in both $\beta 4$ subunits of the segment of residues 115–150 (Fig. 4) and the possible communication with $\alpha 5$ and $\alpha 4$ via the displacement of α -helix H1.

The conformational effects on the $\beta 4$ subunits by peptidic binding at $\beta 5$ opens a possible allosteric pathway between the $\beta 5$ subunits. Interestingly, positive cooperativity of the chymotryptic ($\beta 5$'s) activity is consistent with this proposal (24, 26, 27, 32).

In regard to the structural rearrangements of α -helix H1, our observation is also in line with the reported importance of this helix in the global motions and allosteric communication observed in the archaeal 20S (30) and in the HsIV protease (33), the prokaryotic homolog of the eukaryotic proteasome (34, 35).

Fig. 5. Intersubunit propagation of ligand-induced $\beta 5$ structural changes. (A) $C\alpha$ difference of $\beta 5$ between the apo structure (1RYP) and the average non-peptidic complexes (black); average peptidic complexes with $\beta 5$ specific ligands (blue), and average peptidic complexes with ligands also in other active subunits (red). Dashed lines and shaded regions presented as in Fig. 4. The colored bars on top highlight contact regions with neighboring subunits. The bold numbers indicate five segments, distal from Thr1, where high variations are observed. (B) Subunits surrounding $\beta 5$ (black structure) with H1 α -helix highlighted (red dashed ellipsoid). (C) PCA maps considering the contribution of the main chain atoms of the indicated residues. The five distal segments consist of residues 5–14, 30–41, 101–111, 138–154, and 179–186, respectively. In each map, numbers on the right upper and lower corners indicate the number of atoms involved in the analysis and the distance in the PC1 axis between the closest elements of each cluster, respectively. Colored coding is according to Fig. 1. (D) The backbone displacement of segment 4 is shown for $y\beta 5$, $m\beta 5c$, and $m\beta 5i$. The structures are colored as in Fig. 2. The arrows highlight the displacement occurring in $y\beta 5$ and $m\beta 5c$ on peptide binding. The dashed circle highlights the backbone of residues S141, R142, and S142 of $y\beta 5$, $m\beta 5c$, and $m\beta 5i$, respectively.

The results from the HslV protease (33, 36, 37), thermoplasma acidophilum 20S (30, 31), murine 20S (14), and the here reported murine and yeast 20S analysis suggest that the displacement of the α -helix H1 is a common allosteric trigger linking the status of the active site with the entry gate into the proteasome with diverse functional consequences (28, 29).

This question led us to analyze available structural data on the yCP-open gate mutant where the N-terminal segment of the $\alpha 3$ subunit, which is central in the entangled structure of the entry port, had been deleted (38). This mutation causes structural disorder of the entry pore, which seals the particle lumen in the WT species, thus opening an axial channel into the proteolytically active inner chamber and displaying strongly enhanced peptidase activity. For that same reason, a comparison with the proteasome component of the complexes with the 11S activator and Blm10, respectively, which also display an open entry port, was added (39, 40). Both molecular structures are unliganded and lie in the apo-cluster region of the PCA, failing to provide structural evidence for a reciprocal signal from the gate to the active site in the sense of the described structural alterations in $\beta 5$. Other signals that escaped our analysis may exist, and we are aware that possible conformational shifts can be suppressed by constraints of the crystal lattice.

The discovery of two clusters of CP conformers and their specific structural differences related to peptidic ligand binding in the yeast system cannot, to our knowledge, offer a functional correlation in the sense of enhanced affinity or binding rates of nonpeptidic ligands that do not induce/require structural changes in $\beta 5$, because of the lack of strictly comparable pairs of ligands. However, recent work (41) showed substantial discrimination between cCP and iCP and specificity for cCP by a nonpeptidic inhibitor in accordance with the structural features described here, but the contribution of different side chain interactions cannot be singled out.

The mammalian system, however, offers a conspicuous correlation between structural and functional data. The binding of

a peptidic ligand causes domain closure and movements of about 1 Å in $y\beta 5$ and similarly in $m\beta 5c$ (Fig. 2). In contrast, $m\beta 5i$ is in a preformed configuration optimal for peptidic ligand (and presumably substrate) binding in the apo state. The domains are closed and geared up for peptide binding without requiring domain motion. These observations suggest that the formation of the antiparallel β -sheet when the ligand peptide aligns with protein segments containing residues T21, G47, and A49 is the principal driving force for the overall change of the $\beta 5$ backbone, which is not restricted to neighboring protein segments, but affects the entire subunit in various ways. The energies associated with these structural transitions of $\beta 5$ and their propagation to the adjacent subunits $\beta 4$ and $\beta 6$ are difficult to evaluate. It is obvious, however, that they can contribute to the activation energy of ligand binding and may be major factors for the observed enhanced activity of the immune proteasome mentioned earlier.

The presented study is relevant for pharmacology and specifically for advanced design of ligands that discriminate between cCP and iCP. There is experimental evidence for reduced toxicity of specific immune proteasome ligands and added benefits in the therapy of autoimmune disorders. We suggest that inhibitors displaying the characteristic main chain bonding scheme of peptidic ligands have a genuine preference for the iCP. Certainly, side chain and subsite interactions also have a major impact in binding affinity and selectivity. Moreover, our findings suggest that assisting or impeding the backbone shift of the distal segments may have significant effects on affinity and kinetics of peptidic inhibitors. The design of external binding inhibitors distant from the active site therefore might emerge as an option. In either case, molecular dynamics simulations, as shown here, can be used to guide design and experiments.

Methods

Structural Classification of Reported PDB Structures. Currently the RCSB protein databank (42) lists 46 X-ray crystal structures of yCP together with four

murine and one bovine CPs. This ensemble, including the here reported structure in complex with the Boc-(Ala)₃-al inhibitor, constitutes the currently available high-resolution structural information (Tables S1 and S4). The structures belong to two disjoint classes: structures with the active site occupied by peptidic inhibitors and structures in the apo-state or in complex with nonpeptidic inhibitors. We define a CP ligand as peptidic if it aligns antiparallel in between protein segments and establishes the corresponding hydrogen bonds with Thr21N, Thr21O, Gly47O, and Ala49N (Fig. 2A), regardless of a covalent bond with –Thr1.

PCA. PCA is a statistical tool that has been successfully applied to identify protein domain motion (17–19). Given an ensemble of structures, the algorithm consists of generating an average structure and using it as a common reference for calculating a covariance matrix

$$c_{ij} = \langle (x_i - \langle x_i \rangle) (x_j - \langle x_j \rangle) \rangle. \quad [1]$$

The matrix elements c_{ij} contain averaged information on the correlated deviations of the atomic coordinates, x_i and x_j , from the corresponding

ensemble average, $\langle x_i \rangle$ and $\langle x_j \rangle$. Diagonalization of C provides a space transformation to represent these correlations in a set of orthogonal vectors, thereby dissecting the global displacements into independent components, and is performed by solving the eigenvalue problem

$$A^T C A = \lambda. \quad [2]$$

The matrix A represents the eigenvectors and λ represents the associated eigenvalues. The magnitude of the eigenvalues reflects the magnitude of the displacements described by its associated eigenvectors. We performed a PCA analysis on the ensemble of $\beta 5$ yeast crystal structures (Table S1 and SI Methods) using the GROMACS (43) subroutines `g_covar` and `g_anaeig`.

MD Simulations. The MD simulations were performed using the GROMACS (43) (v. 4.6.2) molecular simulation package (SI Methods).

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