Supplementary Materials

Cooperative binding of the cationic porphyrin Tris-T4 enhances catalytic activity of 20S proteasome unveiling a complex distribution of functional states

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Experimental section.

**NMR spectroscopy.** All NMR spectra were recorded at 298K on a Varian Inova 600 MHz spectrometer, with a 5-mm inverse-detection cryoprobe equipped with z-gradient, located at IBB-CNR, Napoli.

The 1H NMR signal assignment of **Tris-T4** was obtained from the analysis of the spectra assisted by the theoretical prediction based in the molecular structure using ChemAxon software (http://www.chemaxon.com).

For interaction studies, samples (total volume, 250 μl) were prepared in 3 mm NMR tubes, using 0.860 mM of 20S and 175 mM of **Tris-T4** in 50 mM Hepes, 100 mM NaCl and 1mM DTT; ligand/20S molar ratio of 200:1 was used.

STD spectra were acquired with 512 scans with on-resonance irradiation at −0.5 ppm for selective saturation of protein resonances and off-resonance irradiation at 30 ppm for reference spectra. A train of 40 Gaussian-shaped pulses of 50 ms with 1 ms delay between pulses were used, for a total saturation time of 2 s. STD spectra were obtained by internal subtraction of the saturated spectrum from the reference spectrum by phase cycling with a spectral width of 7191.66 Hz, relaxation delay 1.0 s, 8k data points for acquisition, and 16k for transformation. WaterLOGSY NMR experiments employed a 20 ms selective Gaussian 180° pulse at the water signal frequency and a NOE mixing time of 1 s. Both for STD and WaterLOGSY, the FIDs were multiplied by an exponential weighting (lb = 5 Hz) before Fourier transformation.

1H and STD spectra of **Tris-T4** alone were acquired as reference.

1D 1H, STD and WaterLOGSY spectra were processed by means of the ChemAxon software (http://www.chemaxon.com)

The STD factor was calculated as $A_{STD} = \frac{(I_0 - I_{sat})}{I_0} = \frac{I_{STD}}{I_0}$ where $I_0$ is the intensity of the signal in the reference experiment and $I_{sat}$ is the intensity of the same signal in the saturated spectrum. The signal obtained with the strongest $I_{STD}/I_0$ value was normalized to 100%. The relative degree of saturation for the individual protons was used to compare the STD effect.
Calculation of the chemical-physical properties of Tris-T4. The apparent pKa values of Tris-T4 were estimated by using the algorithm ACD/pKa GALAS (ACD/Percepta software, version 2017.1.3, Advanced Chemistry Development, Inc., Toronto, ON, Canada, 2017, [http://www.acdlabs.com](http://www.acdlabs.com)). The compound was considered in its cationic form in all calculations performed, as a consequence of the estimation of percentage of neutral/ionized forms computed at pH 7.2 (cytoplasmic value) using the Handerson–Hasselbalch equation. Atomic potentials were assigned using the CVFF force field, while the partial charges were assigned using the partial charges estimated by MNDO semi-empirical 1 SCF calculations.

The conformational space of the porphyrin was sampled through 200 cycles of simulated annealing (SA; ε =80*r) followed by molecular mechanics (MM) energy minimization. During the SA procedure, the temperature is altered in time increments from an initial temperature to a final temperature by adjusting the kinetic energy of the structure (by rescaling the velocities of the atoms). The following protocol was applied: the system was heated to 1000 K over 2000 fs (time step of 1.0 fs); a temperature of 1000 K was applied to the system for 2000 fs (time step of 1.0 fs) to surmount torsional barriers; successively, temperature was linearly reduced to 300 K in 1000 fs with a decrement of 0.5 K/fs (time step of 1.0 fs). Resulting conformations were then subjected to MM energy minimization within Insight 2005 Discover 3 module (CVFF force field; ε = 80*r) until the maximum rms was less than 0.001 kcal/Å, using conjugate gradient as the minimization algorithm. The resulting MM conformers were subsequently ranked by: i) conformational energy (ΔE from the global energy minimum < 5 kcal/mol), ii) interatomic distances between the charged nitrogen atoms, and iii) conformation of porphyrin ring. In order to properly analyze the electronic properties, the conformers, obtained from molecular dynamics and mechanics calculations, were subjected to a full geometry optimization through semiempirical calculations, using the quantum mechanical method PM7 in the Mopac2012 package (Stewart Computational Chemistry: Colorado Springs, CO, USA; [http://OpenMOPAC.net](http://OpenMOPAC.net) (2012). The EF (Eigenvector Following routine) algorithm of geometry optimization was used, with a GNORM value set to 0.01. To reach a full geometry optimization, the criterion for terminating all optimizations was increased by a factor of 100, using the keyword PRECISE.
The resulting PM7 conformers were subsequently ranked as reported above for MM conformers. The global minimum conformer of Tris-T4 was selected for the dynamic docking studies in complex with human 20S.

**Docking studies on human 20S proteasome.** Docking calculations were performed by using our previously developed atomic models of human 20S in the closed and open conformational states. It has to be underlined that, since we used as template for human 20S in the open state the structure determined by Chen et al. by cryo-electron microscopy (cryoEM), then, our atomic model of human 20S in the open state is composed by just one “half” (one -ring and one -ring) of the entire 20S structure.

The four starting structures to be used in docking studies were generated by positioning: i) one Tris-T4 molecule at α5-α6 groove (closed state); ii) one Tris-T4 at α4-α5 (open state); iii) three Tris-T4 molecules bound at α1-α2, α4-α5 and α5-α6 grooves (closed and open states). The starting complexes were then subjected to dynamic docking studies (Affinity, SA_Docking; Insight2005, Accelrys, San Diego). In particular, a docking methodology, which considers all the systems flexible (i.e., ligand and protein), was used. Flexible docking was achieved using the Affinity module in the Insight 2005 suite, setting the SA_Docking procedure and using the Cell Multipole method for non-bonded interactions.

The docking protocol included a Monte Carlo based conformational search of the ligand within the obtained homology models of human 20S proteasome (i.e., closed and open conformation) for the random generation of a maximum of 20 acceptable complexes. During the first step, in the starting structures, the ligand was moved by a random combination of translation, rotation, and torsional changes to sample both the conformational space of the ligand and its orientation with respect to the binding domain area (MxRChange = 3 Å; MxAngChange = 180°). The binding domain area was defined as a subset including all residues of human 20S proteasome. Thus, all proteasome atoms were left free to move during the entire course of docking calculations, whereas, in order to avoid unrealistic results during the subsequent SA calculations, a tethering restraint was applied on the SCRs of the protein (defined below).
During the Monte Carlo/Metropolis docking step, van der Waals (vdW) and Coulombic terms were scaled to a factor of 0.1 to avoid very severe divergences in the vdW and Coulombic energies. If the energy of a complex structure resulting from random moves of the ligand was higher by the energy tolerance parameter than the energy of the last accepted structure, it was not accepted for minimization. To ensure a wide variance of the input structures to be successively minimized, an energy tolerance value of $10^6$ kcal/mol from the previous structure was used. After the energy minimization step (conjugate gradient; 2500 iterations; $\varepsilon = 1$), the energy test, with an energy range of 50 kcal/mol, and a structure similarity check (rms tolerance = 0.3 kcal/Å) was applied to select the 20 acceptable structures. Each subsequent structure was generated from the last accepted structure. Following this procedure, the resulting docked structures were ranked by their conformational energy and were analyzed considering the non-bonded interaction energies between the ligand and the enzyme (vdW and electrostatic energy contribution; Group Based method\textsuperscript{9}; CUT_OFF = 100; $\varepsilon = 2*r$; Discover_3 Module of Insight2005).

The Monte Carlo docked complexes were then subjected to molecular dynamics simulations at flexible temperatures (Simulated Annealing, SA) to enhance the fixing of the ligand into the binding site and to explore possible ligand-induced large-scale conformational changes of the protein. In particular, the resulting docked complexes were subjected also to a molecular dynamics SA protocol using the Cell_Multipole method for non-bonded interactions and the dielectric constant of the water ($\varepsilon = 80*r$). A tethering restraint was applied on the SCRs of the complex. The set of structural restraints applied was the same as for previous docking calculations. The protocol included 5 ps of a dynamic run divided in 50 stages (100 fs each) during which the temperature of the system was linearly decreased from 500 to 300 K (Verlet velocity integrator; time step = 1.0 fs). In simulated annealing, the temperature is altered in time increments from an initial temperature to a final temperature. The temperature is changed by adjusting the kinetic energy of the structure (by rescaling the velocities of the atoms). Molecular dynamics calculations were performed using a constant temperature and constant volume (NVT) statistical ensemble, and the direct velocity scaling as temperature control method (temp
window = 10 K). In the first stage, initial velocities were randomly generated from the Boltzmann distribution, according to the desired temperature, while during the subsequent stages initial velocities were generated from dynamics restart data. The temperature of 500 K was applied with the aim of surmounting torsional barriers, thus allowing an unconstrained rearrangement of the “ligand” and the “protein” binding site (initial vdW and Coulombic scale factors = 0.1). Successively temperature was linearly reduced to 300 K in 5 ps, and, concurrently, the vdW and Coulombic scale factors have been similarly increased from their initial values (0.1) to their final values (1.0). A final round of 10^5 minimization steps (ε = 80*r) followed the last dynamics steps, and the minimized structures were saved in a trajectory file. The ligand/enzyme complexes thus obtained were ranked by their conformational energy and analyzed considering the non-bonded interaction energies between the ligand and the enzyme (vdW and electrostatic energy contribution; Group Based method; CUT_OFF = 100; ε = 2*r; Discover_3 Module of Insight2005). The complex with the best compromise between the non-bonded interaction energies obtained by Monte Carlo and SA calculations was selected as the structure representing the most probable binding mode. In order to allow the whole relaxation of the protein, the selected annealed complexes were then subjected to MM energy minimization without restraints (Steepest Descent algorithm; ε = 80*r) until the maximum RMS derivative was less than 0.5 kcal/Å (Module Discover; Insight 2005). The protein structural quality in the resulting complexes was then checked using Molprobity structure evaluator software\textsuperscript{10} and compared to that of the reference PDB structure.

The SCRs of the human 20S proteasome were identified using the Structure Prediction and Sequence Analysis server PredictProtein (http://www.predictprotein.org/) and are reported in Table S15. Within the identified SCRs, the following restraints were used: the distance between backbone hydrogen bond donors and acceptors in the alpha-helices was restrained within 2.5 Å. On the other hand, the φ and ψ torsional angles of the beta-sheets were restrained to -119° and +113°, or -139° and +135°, respectively, according to the presence of a parallel or anti-parallel structure. In particular, according to the reliability index values obtained from the secondary structure prediction, the following set
of force constant values were applied (quadratic form): i) 1 kcal/mol/Å² (maximum force: 10 kcal/mol/Å²) for reliability index values from 0 to 3, ii) 10 kcal/mol/Å² (maximum force: 100 kcal/mol/Å²) for reliability index values from 4 to 6, and iii) 100 kcal/mol/Å² (maximum force: 1000 kcal/mol/Å²) for reliability index values from 7 to 9.

**Structural investigation of docked complexes.** In order to evaluate proteasome structure atomic position variations upon TrisT4 binding Root Mean Square Deviations (RMSD) were calculated on Cα atoms by comparing each structure to a reference structure: both the closed and the open conformation were used as reference structure. To this end proteasome structures from all docking calculations including starting conformations were collected and analyzed (39 frames; see Tables 3-6SI). RMSDs were computed taking into account both α and β rings and performed using GROMOS (GROningen MOlecular Simulation computer program package; www.gromos.net).

Structural analyses of the docked complexes were performed using Macromolecules and Receptor-Ligand Interaction tools of Discovery Studio 2017 (Dassault Systèmes BIOVIA, San Diego, 2017). Sequence alignments of yeast and human 20S proteasome subunits were performed using PROMALS3D server (http://prodata.swmed.edu/promals3d/promals3d.php). In order to identified the negatively charged ionic residues of h20S involved in the interaction with the RPs and the hydrophobic residues of h20S involved in the interaction with the C-terminal tails of RPs, the previous structural analysis was implemented using the same procedure on the new experimentally determined structures of h20S proteasome in complex with the regulatory particle 19S (PDB ID: 6MSB, 6MSD, 6MSE, 6MSG, 6MSH, 6MSJ and 6MSK) and PA200 (PDB ID: 6WKY and 6REY). These structures were downloaded from the Protein Data Bank (PDB; http://www.rcsb.org/pdb/) and were analyzed using Biopolymer and Homology modules of Insight 2005 (Accelrys, San Diego).

CAVER was used to identify tunnels considered as void pathways leading from a cavity buried in protein core to the bulk solvent. Tunnels were generated selecting as starting point the center of gravity of all residues within 5 Å from the β5-Thr1 (i.e, R19, A20, V31, K33,
A46, G47, G48, A49, C52) (max distance = 3.0 Å and desired radius = 5.0 Å). Tunnels were generated using the following parameters: 0.9 Å for probe radius (minimum radius of the tunnel), 2.0 Å for shell depth (maximal depth of a surface region) and 3.0 Å for shell radius (radius of the shell probe used to define the bulk solvent). Tunnel clustering was performed by the average-link hierarchical algorithm selecting 2.0 Å for the calculation of pairwise tunnel distances (i.e. dissimilarities) as the cutoff able to capture all the representative directions of the identifies tunnels.

Solvent accessible surface area (SASA) calculations were performed using Discovery Studio 2017 (Dassault Systèmes BIOVIA, San Diego, 2017). The SASA of each Tris-T4 molecule bound to h20S in the selected docked complexes (starting either from the open or the closed protein conformation) was calculated and compared to the SASA of the unbound molecule. The rate of SASA decrease was calculated by using Microsoft Excel.

**Table S1.** Calculated pka values and ionic forms of **Tris-T4** (ACD/pKa GALAS; ACD/Percepta software).

| Compound | pka1      | pka2      | Ionic forms at pH 7.2 (%) |
|----------|-----------|-----------|---------------------------|
| **Tris-T4** | 6.6±1.1   | 11.3±1.2  | Tetra-cationic (21)       |
|          |           |           | Tri-cationic (79)         |
Table S2. Identified clusters of three negatively charged amino acids present on the \( \alpha \)-ring surface of human 20S proteasome in closed and open conformation able to interact with Tris-T4 and related inter-residue distances (C\( \alpha \)).

| Groove | Closed state | Open State |
|--------|--------------|------------|
|        | \( d1 (\text{Å}) \) | \( d2(\text{Å}) \) | \( d1 (\text{Å}) \) | \( D2(\text{Å}) \) |
| \( \alpha_1-\alpha_2 \) | 14.07 | 10.41 | 13.23 | 11.72 |
| \( \alpha_4-\alpha_5 \) | E19(\( \alpha_1 \))-E26(\( \alpha_1 \)) | E26(\( \alpha_1 \))-D155(\( \alpha_1 \)) | E19(\( \alpha_1 \))-E26(\( \alpha_1 \)) | E26(\( \alpha_1 \))-D155(\( \alpha_1 \)) |
| \((I)\) | 18.64 | 6.24 | 19.47 | 7.16 |
| \((II)\) | D13(\( \alpha_4 \))-E24(\( \alpha_4 \)) | D13(\( \alpha_4 \))-E29(\( \alpha_5 \)) | D13(\( \alpha_4 \))-E24(\( \alpha_4 \)) | D13(\( \alpha_4 \))-E29(\( \alpha_5 \)) |
| \( \alpha_4-\alpha_5 \) | 13.21 | 6.24 | 12.74 | 7.16 |
| \((I)\) | E20(\( \alpha_4 \))-D13(\( \alpha_4 \)) | D13(\( \alpha_4 \))-E29(\( \alpha_5 \)) | E20(\( \alpha_4 \))-D13(\( \alpha_4 \)) | D13(\( \alpha_4 \))-E29(\( \alpha_5 \)) |
| \( \alpha_5-\alpha_6 \) | 19.54 | 6.10 | - | - |
| \((I)\) | E29(\( \alpha_5 \))-E18(\( \alpha_5 \)) | E18(\( \alpha_5 \))-E27(\( \alpha_6 \)) | - | - |
| \( \alpha_5-\alpha_6 \) | 11.48 | 6.10 | 14.52 | 7.86 |
| \((II)\) | E25(\( \alpha_5 \))-E18(\( \alpha_5 \)) | E18(\( \alpha_5 \))-E27(\( \alpha_6 \)) | E25(\( \alpha_5 \))-E18(\( \alpha_5 \)) | E18(\( \alpha_5 \))-E27(\( \alpha_6 \)) |
Table S3. Summary of Molprobity results obtained for the experimentally determined structures of human 20S proteasome, their full-length atomic models and the docked porphyrin/20S complexes.

| Structure                        | Residues favored regions | Residues allowed regions | Residues outliers | Poor rotamers |
|----------------------------------|--------------------------|--------------------------|-------------------|--------------|
| X-ray (4R3O) (Closed State)      | 94.9%                    | 4.3%                     | 0.8%              | 4.3%         |
| Cryo-EM (5T0J) (Open State)      | 92.1%                    | 7.7%                     | 0.2%              | 0.9%         |
| Molecular model (Closed State)   | 94.8%                    | 4.3%                     | 0.9%              | 4.1%         |
| Molecular model (Open State)     | 91.4%                    | 8.2%                     | 0.4%              | 1.3%         |
| **Tris-T4 Cplx_1**               |                          |                          |                   |              |
| (3 molecules; starting: closed state) | 78.9%                   | 18.1%                    | 3.0%              | 2.1%         |
| **Tris-T4 Cplx_1**               |                          |                          |                   |              |
| (3 molecules; starting: open state) | 80.3%                   | 16.6%                    | 3.1%              | 2.1%         |
| **Tris-T4 Cplx_5**               |                          |                          |                   |              |
| (1 molecule; starting: closed state) | 79.7%                   | 17.4%                    | 2.9%              | 2.1%         |
| **Tris-T4 Cplx_4**               |                          |                          |                   |              |
| (1 molecule; starting: open state) | 77.8%                   | 18.8%                    | 3.4%              | 1.7%         |
Table S4. Nonbonded interaction energies (kcal/mol) of the 20S-Tris-T4 complexes obtained by Monte Carlo and SA calculations using as starting binding site the α5-α6 groove of 20S in the closed conformation.

| Cplx | Nonbonded interaction energies (kcal/mol) |
|------|------------------------------------------|
|      | Monte Carlo Simulation | Simulated Annealing Simulation |
|      | vdW | Coulomb | Total | vdW | Coulomb | Total |
| 1    | -25.469 | -13.735 | -39.204 | -41.669 | -7.209 | -48.877 |
| 2    | -27.596 | -13.286 | -40.883 | -36.189 | -7.482 | -43.672 |
| 3    | -22.867 | -14.792 | -37.659 | -36.410 | -16.741 | -53.151 |
| 4    | -17.667 | -18.392 | -36.059 | -35.085 | -15.979 | -51.064 |
| 5<sup>a</sup> | -23.333 | -14.690 | -38.023 | -51.398 | -4.093 | -55.491 |
| 6    | -0.527  | -2.558  | -3.085  | -33.174 | 0.962  | -32.212 |
| 7    | -8.953  | -7.614  | -16.567 | -51.291 | -6.460 | -57.751 |
| 8    | -24.361 | -14.594 | -38.955 | -47.097 | -2.378 | -49.475 |
| 9    | -33.095 | -6.576  | -39.671 | -56.979 | -7.744 | -64.723 |
| 10   | -25.005 | -10.483 | -35.487 | -43.958 | -0.951 | -44.909 |
| 11   | -0.525  | -2.739  | -3.264  | -43.364 | 9.964  | -33.399 |

<sup>a</sup>Selected complexes
Table S5. Nonbonded interaction energies (kcal/mol) of the 20S-Tris-T4 complexes obtained by Monte Carlo and SA calculations using as starting binding site the α4-α5 groove of 20S in the open conformation.

| Cplx | Nonbonded interaction energies (kcal/mol) |         |         |         |         |         |         |
|------|------------------------------------------|---------|---------|---------|---------|---------|---------|
|      |                                          | Monte Carlo Simulation | Simulated Annealing Simulation |         |         |         |         |
|      |                                          | vDW     | Coulomb | Total   | vDW     | Coulomb | Total   |
| 1    |                                          | -18.783 | -8.860  | -27.643 | -53.566 | 2.540   | -51.025 |
| 2    |                                          | -18.005 | -8.961  | -26.965 | -57.454 | -0.996  | -58.450 |
| 3    |                                          | -16.783 | -11.143 | -27.928 | -42.356 | -9.547  | -51.903 |
| 4^a  |                                          | -16.636 | -10.984 | -27.620 | -51.230 | -9.600  | -60.831 |
| 5    |                                          | -3.187  | -7.140  | -10.327 | -62.339 | -6.105  | -68.444 |
| 6    |                                          | 2.679   | -7.993  | -5.315  | -27.843 | -4.341  | -32.184 |
| 7    |                                          | -5.179  | -3.223  | -8.401  | -56.803 | -2.897  | -59.700 |
| 8    |                                          | -5.508  | -5.007  | -10.514 | -46.283 | -4.819  | -51.103 |
| 9    |                                          | -2.834  | -3.978  | -6.812  | -41.699 | 1.654   | -40.045 |

^aSelected complex
Table S6. Nonbonded interaction energies (kcal/mol) of the 20S in complex with three molecules of Tris-T4 obtained by Monte Carlo and SA calculations using as starting binding sites the α1-α2, α4-α5 and α5-α6 grooves of 20S in the closed conformation.

| Cplx | Nonbonded interaction energies (kcal/mol) |      |      |      | Monte Carlo Simulation |      |      | Simulated Annealing Simulation |      |      |
|------|-------------------------------------------|------|------|------|------------------------|------|------|-------------------------------|------|------|
|      |                                           | vdW  | Coulomb | Total | vdW  | Coulomb | Total |
| 1³   |                                           | -98.800 | -41.517 | -140.317 | -171.108 | 5.795 | -165.313 |
| 2    |                                           | -22.935 | -18.057 | -40.992 | -160.303 | 3.802 | -156.501 |
| 3    |                                           | -78.377 | -35.529 | -113.907 | -157.977 | 10.189 | -147.788 |
| 4    |                                           | -60.500 | -34.477 | -94.977 | -151.422 | -1.875 | -153.297 |
| 5    |                                           | -55.782 | -40.735 | -96.517 | -83.516 | -15.652 | -99.168 |
| 6    |                                           | -77.702 | -15.237 | -92.938 | -120.808 | 13.206 | -106.874 |
| 7    |                                           | -78.997 | -17.388 | -96.385 | -143.897 | 12.652 | -131.245 |
| 8    |                                           | -81.693 | -28.753 | -110.446 | -155.235 | -7.881 | -163.116 |
| 9    |                                           | -66.203 | -17.685 | -83.888 | -161.086 | 4.435 | -156.651 |

³Selected complex
Table S7. Nonbonded interaction energies (kcal/mol) of the 20S in complex with three molecules of Tris-T4 obtained by Monte Carlo and SA calculations using as starting binding sites the α1-α2, α4-α5 and α5-α6 grooves of 20S in the open conformation.

| Cplx | Nonbonded interaction energies (kcal/mol) |
|------|-----------------------------------------|
|      | Monte Carlo Simulation | Simulated Annealing Simulation |
|      | vdW | Coulomb | Total | vdW | Coulomb | Total |
| 1<sup>a</sup> | -66.387 | -18.887 | -85.274 | -144.197 | 3.380 | -140.817 |
| 2    | -4.039 | 7.431 | 3.392 | -116.600 | -5.287 | -121.887 |
| 3    | -5.680 | -0.063 | -5.743 | -143.156 | 7.652 | -135.504 |
| 4    | -9.199 | -3.893 | -13.093 | -131.408 | 22.359 | -109.049 |
| 5    | -1.246 | -5.385 | -6.631 | -40.043 | -11.613 | -51.656 |
| 6    | -3.016 | 4.501 | 1.484 | -89.168 | 12.507 | -76.661 |
| 7    | -15.019 | -3.025 | -18.044 | -133.642 | -10.110 | -143.752 |
| 8    | -2.448 | 3.231 | 0.783 | -145.664 | -12.591 | -158.255 |

<sup>a</sup>Selected complex
**Table S8.** Conformational energies ($\Delta E$ from the Global Minimum Conformer) of **Tris-T4** docked on human 20S proteasome.

| Complex                  | $\Delta E_{GM}$ (kcal/mol) |
|--------------------------|----------------------------|
|                          | TrisT4_1 | TrisT4_2 | TrisT4_3 |
| 20S Closed /1 TrisT4     | 5.54 ($\alpha_5$-$\alpha_6$) | -         | -         |
| 20S Open /1 TrisT4       | -         | 3.70 ($\alpha_4$-$\alpha_5$) | -         |
| 20S Closed /3 TrisT4     | 4.10 ($\alpha_5$-$\alpha_6$) | 3.12 ($\alpha_4$-$\alpha_5$) | 9.48 ($\alpha_1$-$\alpha_2$) |
| 20S Open /3 TrisT4       | 7.73 ($\alpha_5$-$\alpha_6$) | 8.35 ($\alpha_4$-$\alpha_5$) | 10.57 ($\alpha_1$-$\alpha_2$) |
Table S9. Ligand-residue non-bond interaction energies (kcal/mol) of the Tris-T4/h20S complex obtained by Monte Carlo and SA calculations using as starting binding site the α5-α6 groove of 20S in the closed conformation. The residues involved in the interaction with RPs are noted and the RPs are reported. The corresponding wt y20S residues (PROMALS3D alignment; all conserved in the α3ΔN mutant) are also listed.

| h20S amino acids | y20S amino acids | Subunit | Non-bond interaction Energy (kcal/mol) | RPs<sup>b</sup> |
|------------------|------------------|---------|---------------------------------------|-----------------|
| R20<sup>c</sup> | R20              | α5      | -0.545                                | 19S (E<sub>A1</sub>-2, E<sub> Eli</sub>-C2, E<sub>D1</sub>-2) and PA200 |
| V24<sup>c</sup> | V24              | α5      | -1.110                                | 19S (E<sub>A1</sub>-2, E<sub>B1</sub>, E<sub>Eli</sub>-C1 and E<sub>D1</sub>-2) and PA200 |
| E25<sup>c,d</sup> | E25              | α5      | -2.564                                | 19S (E<sub>A1</sub>-2, E<sub>B1</sub>, S<sub>C1</sub>/E<sub>C1</sub>-2 and S<sub>D1</sub>/E<sub>D1</sub>-2), PA28 and PA200 |
| I28<sup>c</sup> | L28              | α5      | -2.889                                | 19S (E<sub>C1</sub>-2 and E<sub>D1</sub>-2) and PA200 |
| E29<sup>d</sup> | E29              | α5      | -7.587                                | 19S (E<sub>A1</sub>-2) |
| A30             | A30              | α5      | -0.500                                | 19S (E<sub>B1</sub>) |
| D157<sup>c</sup> | E158             | α5      | -1.179                                | 19S (E<sub>B1</sub>) and PA200 |
| A169            | A170             | α5      | -1.766                                | 19S (E<sub>C1</sub>-2 and E<sub>D1</sub>-2) and PA200 |
| I170            | I171             | α5      | -0.717                                | 19S (E<sub>A1</sub>-2) |
| G171            | G172             | α5      | -0.138                                | 19S (E<sub>A1</sub>-2) |
| S174            | S175             | α5      | -1.259                                | 19S (E<sub>A1</sub>-2) |
| E175            | E176             | α5      | -10.131                               | 19S (E<sub>A1</sub>-2) |
| G176            | G177             | α5      | -0.637                                | 19S (E<sub>A1</sub>-2) |
| Q178            | Q179             | α5      | -3.957                                | 19S (E<sub>A1</sub>-2) |
| S179            | A180             | α5      | -3.222                                | 19S (E<sub>A1</sub>-2) |
| Q182            | L183             | α5      | -3.400                                | 19S (E<sub>A1</sub>-2) |
| E183            | N184             | α5      | -6.065                                | 19S (E<sub>A1</sub>-2, E<sub>B1</sub>, E<sub>C1</sub>-2, E<sub>D1</sub>-2) and PA200 |
| Q53<sup>c</sup> | A53              | α6      | -2.086                                | 19S (E<sub>A1</sub>-2, E<sub>B1</sub>, E<sub>C1</sub>-2, E<sub>D1</sub>-2) and PA200 |
| S54             | D54              | α6      | -2.117                                | 19S (E<sub>B1</sub>, E<sub>C1</sub>-2, E<sub>D1</sub>-2) and PA200 |
| E55             | E55              | α6      | -3.353                                | 19S (E<sub>B1</sub>, E<sub>C1</sub>-2, E<sub>D1</sub>-2) and PA200 |
| L56             | L56              | α6      | -0.399                                | 19S (E<sub>B1</sub>, E<sub>C1</sub>-2, E<sub>D1</sub>-2) and PA200 |
| Q203            | S205             | α6      | -1.083                                | 19S (E<sub>B1</sub>, E<sub>C1</sub>-2, E<sub>D1</sub>-2) and PA200 |

<sup>a</sup> Both the wild-type and the α3ΔN mutant; the amino acids not conserved between human and yeast 20S proteasome (alignment performed using PROMALS3D server) are evidenced in bold. <sup>b</sup> 19S functional states involved in the reported interaction are specified in brackets. <sup>c</sup>Amino acids of the C-terminal tail of Rpt5 (aa426-aa439; α5/α6) (19S) and PA200 (aa1830-aa1843) having at least one atom within a 4 Å radius from any given h20S residue. <sup>d</sup> Negatively charged residues involved in ionic interaction with RPs (i.e., PA28, PA200 and 19S).
Table S10. Ligand-residue non-bond interaction energies (kcal/mol) of the TrisT4-20S complex obtained by Monte Carlo and SA calculations using as starting binding site the α4-α5 groove of 20S in the open conformation; The residues involved in the interaction with RPs are noted and the RPs are reported. The corresponding wt y20S residues (PROMALS3D alignment; all conserved in the α3ΔN mutant) are also listed.

| h20S amino acids | y20S amino acids<sup>a</sup> | Subunit | Non-bond interaction Energy (kcal/mol) | RPs<sup>b</sup> |
|------------------|-----------------------------|---------|--------------------------------------|----------------|
| D13<sup>c,d</sup> | D14 | α4 | -6.607 | 19S (E<sub>D1</sub>) and PA28 |
| G14<sup>c</sup> | G15 | α4 | -0.917 | |
| H15<sup>c</sup> | H16 | α4 | -0.481 | 19S (E<sub>D1</sub>) |
| E20<sup>c,d</sup> | E21 | α4 | -7.593 | 19S (E<sub>D1</sub>) and PA28 |
| Q23<sup>c</sup> | L24 | α4 | -2.349 | 19S (E<sub>D1</sub>) |
| E24<sup>c,d</sup> | E25 | α4 | -7.463 | 19S (S<sub>A</sub>/E<sub>A1</sub>, S<sub>B</sub>/E<sub>B</sub>, E<sub>D1</sub>) |
| T147 | T150 | α4 | -0.164 | |
| D148<sup>c</sup> | E151 | α4 | -5.178 | 19S (E<sub>D2</sub>) |
| P149 | P152 | α4 | -0.458 | |
| S150<sup>c</sup> | S153 | α4 | -0.878 | 19S (E<sub>D1</sub>) |
| G151 | G154 | α4 | -0.486 | |
| T152<sup>c</sup> | I155 | α4 | -2.562 | 19S (E<sub>D1</sub>) |
| G162 | G165 | α4 | -0.241 | |
| G164 | N167 | α4 | -0.451 | |
| E29<sup>d</sup> | E29 | α5 | -2.363 | 19S (S<sub>A</sub>/E<sub>A1</sub>) |
| I31 | I31 | α5 | -0.715 | |
| K32 | K32 | α5 | -2.271 | |
| L33<sup>c</sup> | L33 | α5 | -5.769 | 19S (E<sub>D1</sub>) |
| G34<sup>c</sup> | G34 | α5 | -2.942 | 19S (E<sub>D1</sub>) |
| S35<sup>c</sup> | S35 | α5 | -0.929 | 19S (E<sub>D1</sub>) |
| T36 | T36 | α5 | -0.630 | |
| E51<sup>d</sup> | E51 | α5 | -4.263 | 19S (S<sub>A</sub>, S<sub>B</sub>) |
| R53<sup>c</sup> | R53 | α5 | -0.153 | 19S (E<sub>D1</sub>) |
| G80<sup>c</sup> | G80 | α5 | -0.148 | 19S (E<sub>D1</sub>) |
| L81<sup>c</sup> | L81 | α5 | -0.088 | 19S (E<sub>D1</sub>) |
| E207<sup>d</sup> | E209 | α5 | -5.963 | 19S (S<sub>A</sub>/E<sub>A1</sub>, S<sub>B</sub>/E<sub>B</sub> and S<sub>C</sub>/E<sub>C1</sub>) |

<sup>a</sup>Both the wild-type and the α3ΔN mutant; the amino acids not conserved between human and yeast 20S proteasome (alignment performed using PROMALS3D server) are evidenced in bold.  
<sup>b</sup>19S functional states involved in the reported interaction are specified in brackets.  
<sup>c</sup>Amino acids of the C-terminal tail of Rpt1 (aa421-aa433; α4/α5) (19S) having at least one atom within a 4 Å radius from any given h20S residue.  
<sup>d</sup>Negatively charged residues involved in ionic interaction with RPs (i.e., PA28, PA200 and 19S).
Table S11. Ligand-residue non-bond interaction energies (kcal/mol) of the 20S in complex with three molecules of Tris-T4 obtained by Monte Carlo and SA calculations using as starting binding sites the α5-α6, α4-α5 and α1-α2 grooves of 20S in the closed conformation. The residues involved in the interaction with RPs are noted and the RPs are reported. The corresponding wt y20S residues (PROMALS3D alignment; all conserved in the α3ΔN mutant) are also listed.

| Molecule   | h20S amino acids | y20S amino acids³ | Subunit | Non-bond interaction Energy (kcal/mol) | RPs² |
|------------|------------------|-------------------|---------|---------------------------------------|------|
| TrisT4_1   |                  |                   |         |                                       |      |
| (α5/α6)    |                  |                   |         |                                       |      |
| F15⁴       | F15              | α5                |         | -0.160                                | 19S  |
| E18⁴       | E18              | α5                |         | -1.182                                | 19S  |
| G19⁴       | G19              | α5                |         | -0.556                                | 19S  |
| L21⁴       | L21              | α5                |         | -0.393                                | 19S  |
| E25⁴,⁵      | E25              | α5                |         | -2.568                                | 19S  |
| E29⁴       | E29              | α5                |         | -4.414                                | 19S  |
| D157⁴      | E158             | α5                |         | -6.356                                | 19S  |
| P158       | P159             | α5                |         | -0.107                                | 19S  |
| S159⁶       | S160             | α5                |         | -1.347                                | 19S  |
| G160       | G161             | α5                |         | -0.316                                | 19S  |
| T161⁴      | T162             | α5                |         | -1.688                                | 19S  |
| F162       | F163             | α5                |         | -0.545                                | 19S  |
| V163       | Y164             | α5                |         | -1.806                                | 19S  |
| A28⁴       | A28              | α6                |         | -0.989                                | 19S  |
| Q31⁴       | Q31              | α6                |         | -3.634                                | 19S  |
| G32⁴       | G32              | α6                |         | -2.111                                | 19S  |
| S33⁴       | S33              | α6                |         | -0.392                                | 19S  |
| A52⁴       | N52              | α6                |         | -0.448                                | 19S  |
| Q53⁴       | A53              | α6                |         | -2.084                                | 19S  |
| G76⁴       | G76              | α6                |         | -0.716                                | 19S  |
| L77⁴       | L77              | α6                |         | -1.810                                | 19S  |
| T78⁴       | A78              | α6                |         | -0.488                                | 19S  |
| A163⁴      | A63              | α6                |         | -1.824                                | 19S  |
| E197       | Q199             | α6                |         | -1.237                                | 19S  |
| T198       | S200             | α6                |         | -1.416                                | 19S  |
| L199       | L201             | α6                |         | -1.518                                | 19S  |
| P200       | R202             | α6                |         | -0.602                                | 19S  |
|       |       |       |       |       |
|-------|-------|-------|-------|-------|
| Q203  | S205  | α6    | -2.517|       |
| D204⁻ |       | α6    | -8.103| PA200 |
| L205  | L206  | α6    | -0.448|       |

**TrisT4_2**

(α4/α5)

|       |       |       |       |       |
|-------|-------|-------|-------|-------|
| Q146  | Q149  | α4    | -0.377|       |
| D148  | E151  | α4    | -2.164|       |
| T152⁻ | I155  | α4    | -0.524| 19S (E01-2) |
| H154  | S157  | α4    | -2.177|       |
| W156⁻ | W159  | α4    | -1.409| 19S (E01-2) |
| A158  | A161  | α4    | -0.497|       |
| N159  | Q162  | α4    | -1.120|       |
| A160  | T163  | α4    | -0.754|       |
| G164  | N167  | α4    | -0.641|       |
| A165⁻ | S168  | α4    | -1.294| 19S (E02) |
| E170⁻ | E173  | α4    | -3.668| PA200  |
| E173  | E176  | α4    | -3.453|       |
| A30   | A30   | α5    | -0.322|       |
| I31   | I31   | α5    | -0.764|       |
| L33⁻  | L33   | α5    | -4.628| 19S (E01-2) |
| G34⁻  | G34   | α5    | -1.624| 19S (E01-2) |
| S35⁻  | S35   | α5    | -0.416| 19S (E01-2) |
| R53⁻  | R53   | α5    | -0.605| 19S (E01-2) |
| T55⁻  | T55   | α5    | -0.468| 19S (E01-2) |
| G80⁻  | G80   | α5    | -0.421| 19S (E01-2) |
| L81⁻  | L81   | α5    | -0.754| 19S (E01-2) |
| I82⁻  | T82   | α5    | -1.766| 19S (E01-2) |
| G171  | G173  | α5    | -0.175|       |
| S172  | S174  | α5    | -2.659|       |
| A173  | G175  | α5    | -1.043|       |
| S174  | S176  | α5    | -0.127|       |
| Q204  | Q206  | α5    | -3.181|       |
| V205  | V207  | α5    | -3.203|       |
| M206  | M208  | α5    | -2.220|       |
| E207⁻ | E209  | α5    | -5.295| 19S (S/A/A1-2; S/o/E and S/c/E1-2) |
| E208  | E210  | α5    | -2.889|       |

**TrisT4_3**

(α1/α2)

|       |       |       |       |       |
|-------|-------|-------|-------|-------|
| G20   | G23   | α1    | -0.647|       |
| L22⁻  | L25   | α1    | -1.152| 19S (E/A1-2; E/a/E1-2; E1-2) |
| V25⁻  | V28   | α1    | -1.116| 19S (E/A1-2; E/a/E1-2; E1-2) |
| E26⁻⁻ | E29   | α1    | -7.734| 19S (E/A1-2; E/a/E1-2; E1-2) and PA28 |
| F29⁻  | F32   | α1    | -8.420| 19S (E/A1-2; E/a/E1-2; E1-2) |
| I32   | T35   | α1    | -0.205|       |
| N33   | N36   | α1    | -2.428|       |
| Q34   | Q37   | α1    | -2.107|       |
| S39   | S42   | α1    | -0.368|       |
| V40   | L43   | α1    | -0.084|       |
| D155⁻ | D158  | α1    | -5.244| 19S (E/A1-2) |
| G158  | G161  | α1    | -0.189|       |
| Y159⁻ | Y162  | α1    | -7.923| 19S (E/A1-2; E/a/E1-2; E1-2) |
| Y160  | Y163  | α1    | -2.433|       |
| C161⁻ | V164  | α1    | -1.978| 19S (E1-2) |
| G162  | G165  | α1    | -0.192|       |
| F163  | Y166  | α1    | -3.024|       |
Both the wild-type and the α3ΔN mutant; the amino acids not conserved between human and yeast 20S proteasome (alignment performed using PROMALS3D server) are evidenced in bold.

b 19S functional states involved in the reported interaction are specified in brackets.

c Amino acids of the C-terminal tail of Rpt5 (aa426-aa439; α5/α6), Rpt1 (aa421-433; α4/α5), Rpt3 (aa407-418; α1/α2) (19S) and PA200 (aa1830-aa1843) having at least one atom within a 4 Å radius from any given h20S residue.

d Negatively charged residues involved in ionic interaction with RPs (i.e., PA28, PA200 and 19S).
Table S12. Ligand-residue non-bond interaction energies (kcal/mol) of the 20S in complex with three molecules of **Tris-T4** obtained by Monte Carlo and SA calculations using as starting binding sites the α5-α6, α4-α5 and α1-α2 grooves of 20S in the open conformation. The residues involved in the interaction with RPs are noted and the RPs are reported. The corresponding *wt y*20S residues (PROMALS3D alignment; all conserved in the α3ΔN mutant) are also listed.

| Molecule | h20S amino acids | y20S amino acids | Subunit | Non-bond interaction Energy (kcal/mol) | RPs b |
|----------|------------------|------------------|---------|---------------------------------------|-------|
| **TrisT4_1 (α5/α6)** | F15<sup>c</sup> | F15 | α5 | -0.737 | 19S (E<sub>c1</sub>) |
|          | F17 | P17 | α5 | -0.669 | 19S (S<sub>A</sub>EA1-2, S<sub>B</sub>E6, S<sub>C</sub>; S<sub>D</sub>ED1-2), PA28 and PA200 |
|          | E18<sup>cd</sup> | E18 | α5 | -1.428 | 19S (E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | G19<sup>c</sup> | G19 | α5 | -1.401 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | R20<sup>c</sup> | R20 | α5 | -0.060 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | L21<sup>c</sup> | L21 | α5 | -2.384 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | V24<sup>c</sup> | V24 | α5 | -3.995 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | E25<sup>cd</sup> | E25 | α5 | -5.458 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | Y26 | Y26 | α5 | -0.542 | 19S (E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | I28<sup>c</sup> | L28 | α5 | -1.409 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | E29<sup>cd</sup> | E29 | α5 | -5.811 | 19S (S<sub>A</sub>EA1-2) |
|          | D157<sup>c</sup> | E159 | α5 | -2.870 | 19S (E<sub>a</sub>) and PA200 |
|          | S159<sup>c</sup> | S161 | α5 | -0.028 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | A169 | A171 | α5 | -0.344 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | I170 | I172 | α5 | -0.170 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | A28<sup>c</sup> | A28 | α6 | -1.582 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | V29 | I29 | α6 | -0.526 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | Q31<sup>c</sup> | Q31 | α6 | -3.956 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | G32<sup>c</sup> | G32 | α6 | -2.158 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | S33<sup>c</sup> | S33 | α6 | -0.340 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | A34<sup>c</sup> | V34 | α6 | -0.107 | 19S (E<sub>c1</sub>-E<sub>d1</sub>-2) |
|          | A52<sup>c</sup> | N52 | α6 | -3.273 | 19S (E<sub>c1</sub>-E<sub>d1</sub>-2) |
|          | Q53<sup>c</sup> | A53 | α6 | -7.749 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | S54 | D54 | α6 | -1.288 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | E55 | E55 | α6 | -7.095 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | L56 | L56 | α6 | -1.559 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | A57 | S57 | α6 | -0.286 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
|          | G76<sup>c</sup> | G76 | α6 | -0.323 | 19S (E<sub>a1</sub>-E<sub>b</sub>-E<sub>c1</sub>-E<sub>d1</sub>-2) and PA200 |
| L77 | L77 | α6 | 19S (E1A-2, E8, E1C-2, E1D-2) and PA200 |
|-----|-----|----|----------------------------------|
| L161 | L161 | α6 | -0.725 | 19S (E1A-2, E8, E1C-2, E1D-2) and PA200 |
| L205 | L206 | α6 | -0.419 | 19S (E1A-2, E8, E1C-2, E1D-2) and PA200 |
| **TrisT4_2** (α4/α5) | | | | 19S (E1D-2) and PA28 |
| D13 | D14 | α4 | -6.862 | 19S (E1D-2) and PA28 |
| G14 | G15 | α4 | -1.351 | 19S (E1D-2) and PA28 |
| H15 | H16 | α4 | -4.261 | 19S (E1D-2) and PA28 |
| L16 | L17 | α4 | -0.813 | 19S (E1D-2) and PA28 |
| F17 | F18 | α4 | -2.093 | 19S (E1D-2) and PA28 |
| V19 | V20 | α4 | -0.309 | 19S (E1D-2) and PA28 |
| E20 | E21 | α4 | -11.329 | 19S (E1D-2) and PA28 |
| Y21 | Y22 | α4 | -5.754 | 19S (E1D-2) and PA28 |
| Q23 | L24 | α4 | -2.031 | 19S (E1D-2) and PA28 |
| E24 | E25 | α4 | -2.093 | 19S (E1D-2) and PA28 |
| Q146 | Q149 | α4 | -5.754 | 19S (E1D-2) and PA28 |

| | | | | 19S (E1D-2) and PA28 |
| A160 | T163 | α4 | -0.267 | 19S (E1D-2) and PA28 |
| S35 | S35 | α5 | -1.202 | 19S (E1D-2) and PA28 |
| T36 | T36 | α5 | -0.817 | 19S (E1D-2) and PA28 |
| V50 | V50 | α5 | -0.228 | 19S (E1D-2) and PA28 |
| E51 | E51 | α5 | -2.093 | 19S (E1D-2) and PA28 |
| S79 | S79 | α5 | -0.860 | 19S (E1D-2) and PA28 |
| G80 | G80 | α5 | -0.144 | 19S (E1D-2) and PA28 |
| I82 | T82 | α5 | -0.079 | 19S (E1D-2) and PA28 |

| **TrisT4_3** (α1/α2) | | | | 19S (E1C-1) |
| C161 | V164 | α1 | -0.123 | 19S (E1C-1) |
| F163 | Y166 | α1 | -1.500 | 19S (E1C-1) |
| A165 | A168 | α1 | -2.026 | 19S (E1C-1) |
| T166 | T169 | α1 | -1.676 | 19S (E1C-1) |
| A167 | A170 | α1 | -2.756 | 19S (E1C-1) |
| A168 | T171 | α1 | -0.628 | 19S (E1C-1) |
| V170 | P173 | α1 | -1.987 | 19S (E1C-1) |
| Q172 | Q175 | α1 | -1.660 | 19S (E1C-1) |
| T173 | Q176 | α1 | -2.202 | 19S (E1C-1) |
| T176 | T179 | α1 | -1.643 | 19S (E1C-1) |
| E180 | E183 | α1 | -3.787 | 19S (E1C-1) |
| Q52 | S52 | α2 | -2.422 | 19S (E1C-1) |
| S54 | S54 | α2 | -2.747 | 19S (E1C-1) |
| E56 | L56 | α2 | -0.999 | 19S (E1C-1) |
| Y57 | M58 | α2 | -2.438 | 19S (E1C-1) |
| D58 | S59 | α2 | -0.888 | 19S (E1C-1) |

Both the wild-type and the α3ΔN mutant; the amino acids not conserved between human and yeast 20S proteasome (alignment performed using PROMALS3D server) are evidenced in bold. 19S functional states involved in the reported interaction are specified in brackets. Amino acids of the C-terminal tail of Rpt5 (aa426-aa439; α5/α6), Rpt1 (aa421-433; α4/α5), of Rpt3 (aa407-418; α1/α2) (19S) and PA200 (aa1830-aa1843) having at least one atom within a 4 Å radius from any given h20S residue. Negatively charged residues involved in ionic interaction with RPs (i.e., PA28, PA200 and 19S).
Table S13. Calculated rate of solvent accessible surface area (SASA) decrease for the phenyl and N-methyl-pyridyl hydrogen atoms of Tris-T4 bound to h20S in the selected docking solutions.

|            | SASA decrease (%) |                  |                  |                  |                  |                  |
|------------|-------------------|------------------|------------------|------------------|------------------|------------------|
|            |                   | Starting h20S conformation: |                  | Starting h20S conformation: |                  |                  |
|            |                   | closed           |                  | open             |                  |                  |
|            |                   | α1-α2            | α4-α5            | α5-α6            | α1-α2            | α4-α5            | α5-α6            |
| para-Phe  |                   | 100              | 0                | 100              | 100              | 100              |
| meta-Phe  |                   | 100              | 48               | 100              | 100              | 81               | 95               |
| ortho-Phe |                   | 75               | 47               | 43               | 50               | 43               | 61               |
| meta N-Me-Pyr |               | 77               | 67               | 42               | 20               | 40               | 64               |
| ortho N-Me-Pyr |               | 80               | 50               | 47               | 31               | 47               | 47               |
Table S14. Salt bridges and charge-assisted hydrogen bonds involving the N-terminals, $\alpha_5$-loop, and $\alpha_6$-loop present in the experimentally determined structures of yeast 20S proteasome, wt closed (PDB ID: 1RYP), wt open (PDB ID: 1Z7Q) and the $\alpha_3\Delta N$ mutant (PDB ID: 1G0U).

| Structure               | Interactions               |
|-------------------------|----------------------------|
| **yeast 20S closed**    | E125$\alpha_5$-R4$\alpha_3$ |
|                         | E125$\alpha_5$-Y123$\alpha_6$ |
|                         | S128 $\alpha_5$-Y123 $\alpha_6$ |
|                         | R122 $\alpha_5$-E130$\alpha_5$ |
|                         | R136$\alpha_5$-D84$\alpha_5$ |
| **yeast 20S open**      | R132 $\alpha_5$-E130$\alpha_5$ |
|                         | R136$\alpha_5$-D84$\alpha_5$ |
|                         | S128 $\alpha_5$-Y123 $\alpha_6$ |
| **$\alpha_3\Delta N$ mutant 20S** | R130$\alpha_5$-D84$\alpha_5$ |
Table S15. Identified structurally conserved regions (SCRs) of the human 20S proteasome using the Structure Prediction and Sequence Analysis server PredictProtein (http://www.predictprotein.org/).

| Subunits | Amino acid numbering | Secondary structure |
|----------|----------------------|---------------------|
| α1       | 13-16                | β-sheet             |
|          | 23-33                | α-helix             |
|          | 38-43                | β-sheet             |
|          | 48-53                | β-sheet             |
|          | 68-72                | β-sheet             |
|          | 76-80                | β-sheet             |
|          | 85-104               | α-helix             |
|          | 111-128              | α-helix             |
|          | 135-143              | β-sheet             |
|          | 150-154              | β-sheet             |
|          | 160-168              | β-sheet             |
|          | 172-183              | α-helix             |
|          | 191-207              | α-helix             |
|          | 215-222              | β-sheet             |
|          | 226-229              | β-sheet             |
|          | 232-243              | α-helix             |
| α2       | 9-13                 | β-sheet             |
|          | 20-30                | α-helix             |
|          | 34-39                | β-sheet             |
|          | 44-49                | β-sheet             |
|          | 66-68                | β-sheet             |
|          | 72-76                | β-sheet             |
|          | 81-100               | α-helix             |
|          | 107-124              | α-helix             |
|          | 131-139              | β-sheet             |
|          | 145-149              | β-sheet             |
|          | 155-163              | β-sheet             |
|          | 167-178              | α-helix             |
|          | 184-198              | α-helix             |
|          | 208-214              | β-sheet             |
|          | 219-220              | β-sheet             |
|          | 223-231              | α-helix             |
| α3       | 10-12                | β-sheet             |
|          | 18-29                | α-helix             |
|          | 33-38                | β-sheet             |
|          | 43-48                | β-sheet             |
| α4       | 66-68 | β-sheet |
|----------|-------|---------|
|          | 72-76 | β-sheet |
|          | 80-100| α-helix |
|          | 107-124| α-helix |
|          | 131-139| β-sheet |
|          | 146-150| β-sheet |
|          | 157-164| β-sheet |
|          | 168-178| α-helix |
|          | 186-200| α-helix |
|          | 211-217| β-sheet |
|          | 224-227| β-sheet |
|          | 230-248| α-helix |

| α4       | 6-10  | β-sheet |
|----------|-------|---------|
|          | 17-27 | α-helix |
|          | 31-36 | β-sheet |
|          | 41-46 | β-sheet |
|          | 62-65 | β-sheet |
|          | 69-73 | β-sheet |
|          | 78-97 | α-helix |
|          | 104-121| α-helix |
|          | 128-136| β-sheet |
|          | 143-147| β-sheet |
|          | 154-161| β-sheet |
|          | 165-176| α-helix |
|          | 183-198| α-helix |
|          | 206-212| β-sheet |
|          | 217-219| β-sheet |
|          | 222-243| α-helix |

| α5       | 11-15 | β-sheet |
|----------|-------|---------|
|          | 22-32 | α-helix |
|          | 36-41 | β-sheet |
|          | 46-51 | β-sheet |
|          | 66-70 | β-sheet |
|          | 74-78 | β-sheet |
|          | 83-102| α-helix |
|          | 109-120| α-helix |
|          | 138-146| β-sheet |
|          | 152-156| β-sheet |
|          | 162-170| β-sheet |
|          | 174-185| α-helix |
|          | 191-206| α-helix |
|          | 215-221| β-sheet |
|          | 226-228| β-sheet |
|          | 231-240| α-helix |
| Region | Type   |
|--------|--------|
| α6     | 10-13  | β-sheet |
|        | 19-30  | α-helix |
|        | 35-39  | β-sheet |
|        | 45-49  | β-sheet |
|        | 62-66  | β-sheet |
|        | 70-74  | β-sheet |
|        | 79-98  | α-helix |
|        | 105-122| α-helix |
|        | 129-137| β-sheet |
|        | 143-147| β-sheet |
|        | 154-161| β-sheet |
|        | 165-176| α-helix |
|        | 184-199| α-helix |
|        | 210-216| β-sheet |
|        | 221-223| β-sheet |
|        | 226-236| α-helix |
| α7     | 13-15  | β-sheet |
|        | 22-32  | α-helix |
|        | 36-41  | β-sheet |
|        | 46-51  | β-sheet |
|        | 67-70  | β-sheet |
|        | 74-78  | β-sheet |
|        | 83-102 | α-helix |
|        | 109-124| α-helix |
|        | 133-141| β-sheet |
|        | 148-152| β-sheet |
|        | 160-166| β-sheet |
|        | 170-180| α-helix |
|        | 187-202| α-helix |
|        | 212-219| β-sheet |
|        | 224-227| β-sheet |
|        | 229-245| α-helix |
| β1     | 2-8    | β-sheet |
|        | 12-16  | β-sheet |
|        | 25-27  | β-sheet |
|        | 34-37  | β-sheet |
|        | 41-45  | β-sheet |
|        | 52-69  | α-helix |
|        | 76-90  | α-helix |
|        | 95-103 | β-sheet |
|        | 110-114| β-sheet |
|        | 120-122| β-sheet |
|        | 124-128| β-sheet |
|        | 132-143| α-helix |
| β2          | 149-166 | α-helix |
|-------------|---------|---------|
|             | 174-180 | β-sheet |
|             | 185-189 | β-sheet |
|             | 191-200 | α-helix |
|             | 2-8     | β-sheet |
|             | 12-17   | β-sheet |
|             | 25-27   | β-sheet |
|             | 33-37   | β-sheet |
|             | 41-45   | β-sheet |
|             | 52-67   | α-helix |
|             | 76-90   | α-helix |
|             | 95-103  | β-sheet |
|             | 109-113 | β-sheet |
|             | 123-127 | β-sheet |
|             | 131-142 | α-helix |
|             | 148-165 | α-helix |
|             | 173-179 | β-sheet |
|             | 184-186 | β-sheet |
|             | 194-201 | α-helix |
|             | 210-213 | β-sheet |
|             | 215-225 | β-sheet |
| β3          | 9-14    | β-sheet |
|             | 18-24   | β-sheet |
|             | 32-34   | β-sheet |
|             | 41-44   | β-sheet |
|             | 48-52   | β-sheet |
|             | 56-76   | α-helix |
|             | 83-97   | α-helix |
|             | 103-111 | β-sheet |
|             | 119-123 | β-sheet |
|             | 134-138 | β-sheet |
|             | 142-152 | α-helix |
|             | 159-175 | α-helix |
|             | 184-190 | β-sheet |
|             | 194-199 | β-sheet |
| β4          | 3-8     | β-sheet |
|             | 12-17   | β-sheet |
|             | 26-27   | β-sheet |
|             | 32-35   | α-helix |
|             | 36-38   | β-sheet |
|             | 42-46   | β-sheet |
|             | 50-70   | α-helix |
|             | 77-93   | α-helix |
|             | 99-107  | β-sheet |
| β5       |                      |       |
|----------|----------------------|-------|
| 114-118  | β-sheet              |       |
| 129-132  | β-sheet              |       |
| 136-147  | α-helix              |       |
| 153-170  | α-helix              |       |
| 178-184  | β-sheet              |       |
| 189-190  | β-sheet              |       |
| 2-8      | β-sheet              |       |
| 12-16    | β-sheet              |       |
| 25-26    | β-sheet              |       |
| 31-34    | α-helix              |       |
| 35-37    | β-sheet              |       |
| 41-45    | β-sheet              |       |
| 49-69    | α-helix              |       |
| 76-91    | α-helix              |       |
| 96-104   | β-sheet              |       |
| 110-114  | β-sheet              |       |
| 120-122  | β-sheet              |       |
| 124-128  | β-sheet              |       |
| 132-143  | α-helix              |       |
| 149-166  | α-helix              |       |
| 174-180  | β-sheet              |       |
| 185-186  | β-sheet              |       |
| 189-200  | α-helix              |       |
| β6       |                      |       |
| 5-6      | β-sheet              |       |
| 11-16    | β-sheet              |       |
| 20-26    | β-sheet              |       |
| 34-36    | β-sheet              |       |
| 42-46    | β-sheet              |       |
| 50-54    | β-sheet              |       |
| 58-77    | α-helix              |       |
| 85-99    | α-helix              |       |
| 105-113  | β-sheet              |       |
| 120-124  | β-sheet              |       |
| 134-138  | β-sheet              |       |
| 142-152  | α-helix              |       |
| 168-184  | α-helix              |       |
| 193-199  | β-sheet              |       |
| 203-210  | β-sheet              |       |
| β7       |                      |       |
| 10-16    | β-sheet              |       |
| 20-25    | β-sheet              |       |
| 33-35    | β-sheet              |       |
| 39-41    | α-helix              |       |
| 42-45    | β-sheet              |       |
| 49-53    | β-sheet              |       |
| Region  | Secondary Structure |
|---------|---------------------|
| 57-77   | α-helix             |
| 85-100  | α-helix             |
| 107-115 | β-sheet             |
| 121-125 | β-sheet             |
| 136-139 | β-sheet             |
| 143-154 | α-helix             |
| 162-179 | α-helix             |
| 187-193 | β-sheet             |
| 198-199 | β-sheet             |
| 207-216 | α-helix             |
Figure S1. Structure of the Tris-T4 reporting $^1$H chemical shift assignments.
Figure S2. a) 1H NMR spectra of Tris-T4 (100 μM) in the presence of α3ΔN γ20S proteasome (500 nM); b) STD and c) WaterLOGSY spectra.
**Figure S3.** Structure of the calculated prevalent (79%) ionic form of Tris-T4 at cytoplasmic pH (7.2).
Figure S4. A-B: Selected docked complex of one **Tris-T4** molecule bound to human 20S (closed conformation). A) Top view: the protein is displayed as Connolly surface and colored in grey except for the negatively charged residues involved in ionic interactions with RPs which are displayed as CPK and colored in red. **Tris-T4** is colored by atom type (C: green; N: blue) and displayed as CPK. B) Close up view of the ligand binding site at the α5-α6 groove: interacting residues (non-bonded interaction energy lower than -2 kcal/mol) are evidenced as CPK and colored: negative = red; polar = orange. C-D: Close up view of the α5-loop interactions pattern of the closed proteasome 20S in the starting conformation (C) and upon one **Tris-T4** molecule binding at the α5-α6 groove (D). The α-ring is rendered in cartoons and labelled; amino acids involved in ionic interactions are displayed in ball and sticks and labelled. α5-L81 at the top of the allosteric pathway between distinct conformational states described by Ruschak et al.\textsuperscript{13} is evidenced in cyan CPK. **Tris-T4** is shown in grey CPK.
Figure S5. Selected docked complex of one Tris-T4 molecule bound to human 20S (open conformation). A) Top view: the protein is displayed as Connolly surface and colored in grey except for the negatively charged residues involved in ionic interactions with RPs which are displayed as CPK and colored in red. Tris-T4 is colored by atom type (C: green; N: blue) and displayed as CPK. B) Close up view of the ligand binding site at the α4-α5 groove: interacting residues (non-bonded interaction energy lower than -2 kcal/mol) are evidenced as CPK and colored: negative = red; positive = blue; polar = orange; hydrophobic = magenta. C-D: Close up view of the α5-loop interactions pattern of the open proteasome 20S in the starting conformation (C) and upon one Tris-T4 molecule binding at the α4-α5 groove (D). α-ring is rendered in cartoons and labelled; amino acids involved in ionic interactions are displayed in ball and sticks and labelled. α5-L81 at the top of the allosteric pathway between distinct conformational states described by Ruschak et al.13 is evidenced in cyan CPK. Tris-T4 is shown in grey CPK.
Figure S6. A-B: Close up view of the ligand binding site at the α1-α2 (A; closed) and α1-α2 (B; open) grooves. Tris-T4 is colored by atom type (C: green; N: blue) and displayed as CPK. The protein is displayed as Connolly surface and colored in gray. The residues involved in interactions with Tris-T4 are displayed in CPK and colored: orange (negative residues involved in ionic interactions with RPs) and cyan (residues not engaged in RP interactions). The residues involved in interactions with RPs hydrophobic motif are evidenced in ball&stick.
Figure S7. A) Dynamic docking results obtained for Tris-T4 using as starting structure the α5-α6 groove of the closed conformation of human 20S proteasome. B) Top view of dynamic docking results at the first α-ring: the first five generated complexes (Table S4) show Tris-T4 bound at the α5-α6 groove (although presenting a different binding mode with respect to the starting position). C) Top view of dynamic docking results without the first ring of α subunits: the last six generated complexes presented the ligand positioned either at the level of the first β-ring or at the interface between the first and the second β-ring (Table S4). The backbone of the starting complex is displayed as solid ribbons and colored in pink (α1), orange (α2), brown (α3), light green (α4), cyan (α5), magenta (α6), and gray (α7, α subunits of the second ring and all β subunits). The backbone of the calculated complexes is displayed as line ribbons and colored in orange (B and C). The porphyrin ligands are colored by atom type (C: green and N: blue) and displayed as CPK. In B the α subunits and the catalytic β subunits are labeled. In C the catalytic β subunits are labeled and colored in pink (β1), violet (β2), and cyan (β5).
Figure S8. A) Dynamic docking results obtained for Tris-T4 using as starting structure the α4-α5 groove of the open conformation of human 20S proteasome. B) Top view of dynamic docking results at the α-ring: all obtained complexes showed Tris-T4 bound at the α4-α5 groove (Table S5), although assuming different binding poses with respect to the starting position. The backbone of the starting complex is displayed as solid ribbons and colored in pink (α1), orange (α2), brown (α3), light green (α4), cyan (α5), magenta (α6), and grey (α7, all β subunits). The backbone of the calculated complexes is displayed as line ribbons and colored in orange (B). The porphyrin ligands are colored by atom type (C: green and N: blue) and displayed as CPK. In B the α subunits and the catalytic β subunits are labeled.
Figure S9. A) Dynamic docking results obtained for Tris_T4 using as starting points the α1-α2, α4-α5, and α5-α6 grooves of the closed conformation of human 20S proteasome. B) Top view of dynamic docking results without the two rings of β subunits and the second ring of α subunits. C) Top view of dynamic docking results without the first ring of α subunits. The analysis of the whole of the generated complexes using the 20S closed conformation as starting structure showed that in four of them (2-5 in Table S6) one molecule of Tris-T4 is placed at the level of the first β-ring (A and C). The other two molecules are localized on the α-ring, with one of the two always positioned at α5-α6 groove (B). Finally, in the subsequent four complexes (6-9 in Table S6), all three molecules move down, placing at the interface of the β rings or between the α5 and α6 subunits.

The backbone of the starting complex is displayed as solid ribbons and colored in pink (α1), orange (α2), brown (α3), light green (α4), cyan (α5), magenta (α6), and grey (α7, α subunits of the second ring and all β subunits). The backbone of the calculated complexes is displayed as line ribbons and colored in orange (B and C). The porphyrin ligands are colored by atom type (C: green and N: blue) and displayed as CPK. In B the α subunits and the catalytic β subunits are labeled. In C the catalytic β subunits are labeled and colored in pink (β1), violet (β2), and cyan (β5).
**Figure S10.** A) Dynamic docking results obtained for Tris_T4 using as starting points the α1-α2, α4-α5, and α5-α6 grooves of the open conformation of human 20S proteasome. B) Top view of dynamic docking results without the ring of β subunits. C) Bottom view of dynamic docking results without the ring of β subunits. The ligand molecule bound to the α1-α2 groove moves down the interior of the α-ring or toward the center of the protein (A and C). On the other hand, the other two molecules of Tris-T4 in most solutions bind at α4-α5 and α5-α6 grooves although in some binding poses occupying the substrate channel between the α1 and the β-ring or moving along the grooves toward the substrate gate. Interestingly, in four solutions one Tris-T4 binds the negatively charged α5-loop (B). The backbone of the starting complex is displayed as solid ribbons and colored in pink (α1), orange (α2), brown (α3), light green (α4), cyan (α5), magenta (α6), and grey (α7, α subunits of the second ring and all β subunits). The backbone of the calculated complexes is displayed as line ribbons and colored in orange (B and C). The porphyrin ligands are colored by atom type (C: green and N: blue) and displayed as CPK. In B the α subunits and the catalytic β subunits are labeled.
Figure S11. Solvent accessible surface (SASA) of the three Tris-T4 molecules docked to the closed (A) and open (B) h20S conformation (view: from the interior to the exterior of the protein). SASA is coloured in cyan and displayed as solid surface; ligand molecules are coloured by atom types (C = green; N = blue; H = white).
Figure S12. A) RMSD of the Tris-T4/20S complexes generated upon MC/SA docking calculations carried out on the closed (left) and open (right) states with respect to the starting protein conformation. Squared spots indicate single molecule docking (S on x-axis), circled spots indicate triple molecule docking (T on x-axis). Blue colour indicates complexes where at least one ligand molecule occupies superficial binding poses (α surface) whereas red colour is used for complexes where all ligand molecules occupy internal sites (α/β interface. β subunits). Full coloured spots specify selected binding poses. B) RMSD evolution is calculated with respect to the opposite 20S conformation (closed vs. open and open vs. closed; red line) and compared to those obtained using as reference structure the starting conformation (black line), as an index of divergence/similarity from the open/closed starting states. RMSDs are calculated on Cα atoms of the full-length (upper α and β rings) proteasome.
Figure S13. Top view of the human 20S proteasome structure in the closed (A, C; PDB ID: 4R3O) and open (B, D; PDB ID: 5T0J) conformation. A and B: the protein is displayed as Connolly surface and colored in gray; the negatively charged residues involved in ionic interactions with RPs (i.e., 19S, PA28α and PA200) are displayed as CPK and colored in red. C and D: the protein is displayed in ribbons and secondary structure elements of each subunit are sequentially colored from N- to C-terminal end (αH0= blue; αH1= cyan; αH2= magenta, αH3-αH5= light red to dark red).
Figure S14. wt-yeast and α3ΔN-yeast 20S proteasome. Top-view of the α-ring (left) and close-up view of the gate (right). A) wt yeast 20S proteasome structures in closed state (access code: 1RYP); B) wt yeast 20S proteasome structures in open state (access code: 1Z7Q) and in C) mutant form α3ΔN (access code: 1G0U) are shown in line cartoons. Purple ellipses define the proteasome gate and the α5 loop is indicated and labelled. Amino acids of the N-terminal tails involved in the major interactions with the α5 loop are displayed in ball and sticks and labelled, negatively and positively charged residues are coloured red and blue, respectively.
**Figure S15.** Multiple sequence alignment of α5-loop (A) and α6-loop (B) of human, *yeast* and α3ΔN *yeast* 20S. Amino acids establishing salt bridges and charge-assisted hydrogen bonds are evidenced in cyan. Amino acids not conserved in the α3ΔN mutant are evidenced with black boxes.
Figure S16. Structural overlap of the PA200 C-terminal tail with Tris-T4 obtained by fitting the Cα atoms of the 20S α-ring of the Tris-T4/h20S best-docked complexes (three ligand molecules; h20S starting conformation: closed (A) and open (B)) on the Cα atoms of the 20S α-ring of the h20S structure in complex with PA200 (PDB ID: 6KWY). The PA200 C-terminal residues overlapping with Tris-T4 are displayed in ball&stick and labelled (residues having at least one atom within a 2 Å radius from any given Tris-T4 atom). For sake of clarity, only the h20S structure of the docked complex is displayed. Residues interacting with both Tris-T4 and the PA200 residues are displayed in stick and coloured in pink. The vdW volume of L77 (α6) is shown in magenta.
**Figure S17.** Graphical representation of standard Gibbs free energy difference (-ΔG0, as defined in the Experimental section) among the various Tris-T4- and substrate-bound states of h20S. The y-axis values (kJ/mol) are arbitrarily chosen. Circles represent different conformational states and the dimension is indicative of their abundance.
Figure S18. Standard Gibbs free energy difference (-ΔG°, as defined in the Experimental section) among the various Tris-T4 and substrate-bound states of y20S wt and y20S α3ΔN mutant. The y-axis values are in kJ/mol arbitrarily chosen, but the energy differences correspond to those resulting from parameters reported in Table 1. Circles represents different conformational states and the dimension is indicative of their abundance.
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