Supporting Information

TROSY-based NMR evidence for a novel class of 20S proteasome inhibitor

Remco Sprangers, Xiaoming Liqq, Xinliang Mao, John L. Rubinstein, Aaron D. Schimmer and Lewis E. Kay

Supporting Methods

Protein Cloning, Expression and Purification. The monomeric α ring, denoted by α_7 in the text, has been constructed by deleting residues 97 to 103 from the native protein sequence, using conventional cloning techniques. The 20S proteasome was expressed as described previously (1). Briefly, the α and β subunits were either co-expressed from one plasmid (where the β subunit carried a C-terminal His6-tag), co-expressed from 2 different plasmids (where the α subunit contained a TEV cleavable N-terminal His6-tag) or both subunits were expressed separately (with a TEV cleavable His6-tag on each of the subunits). Methyl labeling of proteins was achieved by growing cells in D_2O based minimal medium with ^{2}H-^{12}C glucose as the carbon source. One hour before induction of protein expression, α-ketobutyric acid (60 mg/liter) and α-ketoisovaleric acid (100 mg/liter; only one of the two isopropyl methyls was $^{13}CH_3$ labeled, the other $^{12}CD_3$) were added to introduce $^{13}CH_3$ methyl groups in Ile, Leu and Val (2). Proteins were purified by Ni-NTA affinity resin and size exclusion chromatography. Proteasomes only isotopically enriched in the α subunits were reconstituted by mixing separately expressed α and β subunits followed by an additional size exclusion purification step. Highly deuterated,

$^{15}N/\ ^{13}C^{15}N$ labeled α_7 samples were prepared after first dialyzing the GuHCl (6.0 M) denatured protein against a non-denaturing buffer. NMR samples were typically 300 μM in total proteasome (monomer concentration) in 100% D_2O (^{13}C labeling), 25 mM potassium phosphate pH 6.8, 50 mM NaCl, 1 mM EDTA, 0.03% NaN_3 and 2 mM DTT or in 90%H_2O/10%D_2O ([α_7]; $^{15}N/\ ^{13}C^{15}N$ labeling) with the same buffer composition as above.

Electron Microscopy. Purified α rings (4 μL) at a concentration of 5 μg/mL were applied to the surface of a continuous carbon film coated electron microscopy (EM) grid that had been previously made hydrophilic by glow discharge in air. Protein was allowed to adsorb to the carbon surface for 2 min. The grid was washed once with water (50 μL) and stained with 2 % (w/v) uranyl acetate (50 μL). The grid was imaged with a FEI F20 microscope (FEI Company, Eindhoven, Netherlands) operating at 200 kV with a magnification of 50,000 x and a defocus of 0.5 μm. Images were recorded on Kodak SO-163 film, developed in full strength D19 solution for 12 minutes and digitized with an Intergraph Photoscan densitometer (Intergraph, Huntsville, Alabama, USA) using a 7 μm step size. Pixels were averaged 3 x 3 to give an effective pixel size of 4.2 x 4.2 Å. 1000 particle images were selected interactively, band pass filtered between 700 and 20 Å with a modified Gaussian edge filter and subjected to reference-free alignment with SPIDER (3). Symmetry was not applied during the alignment and averaging. Multivariate statistical analysis with SPIDER failed to provide evidence of any symmetry besides a 7-fold rotation axis.

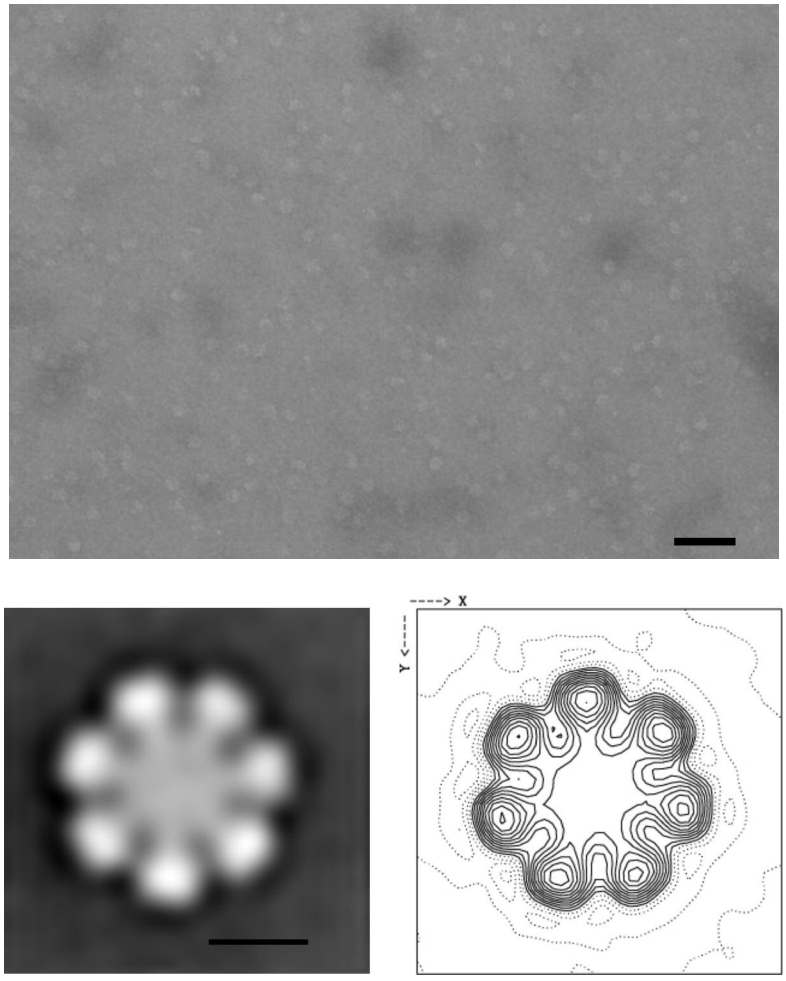

Figure S1. Top: Sample region of an EM micrograph. The scale bar represents 500 Å. Bottom: An average of 1000 aligned images of proteasome α rings and corresponding contour map. The scale bar represents 50 Å.

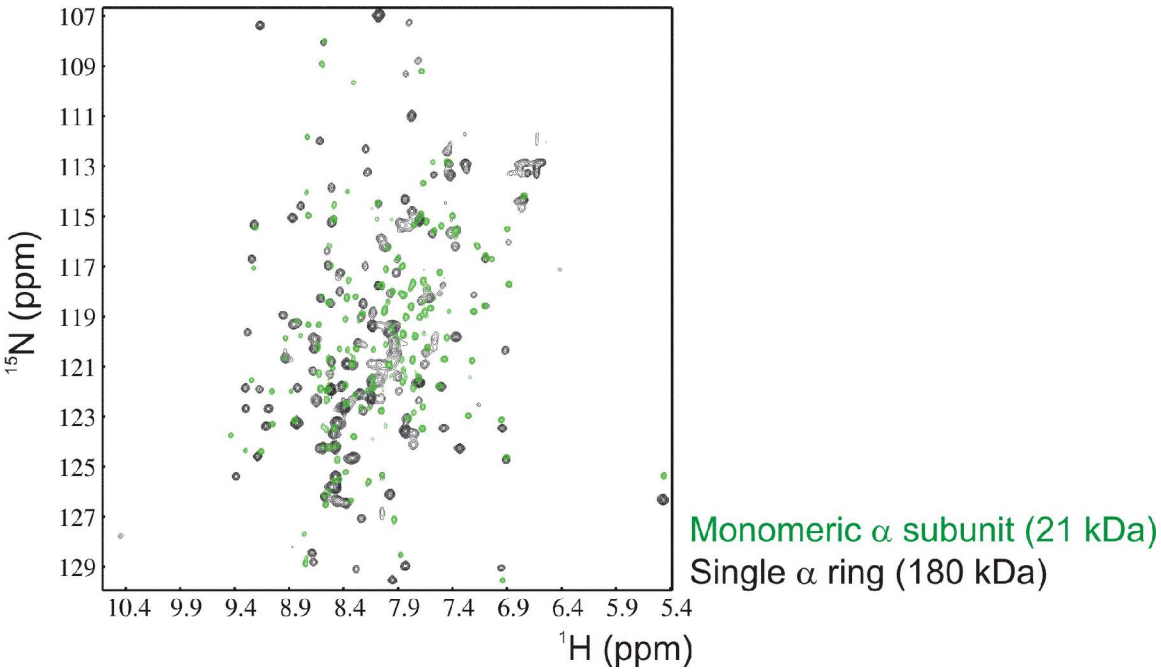

Figure S2. Overlay of the ^{1}H-^{15}N TROSY-HSQC spectra of α_7 (180 kDa, black) with the monomeric alpha subunit (21 kDa, green), 50^{o}C, 600 MHz. A direct transfer of assignments from the spectrum of the single subunit to the spectrum of α_7 is not possible; however, shifts between the two forms are small in many cases.

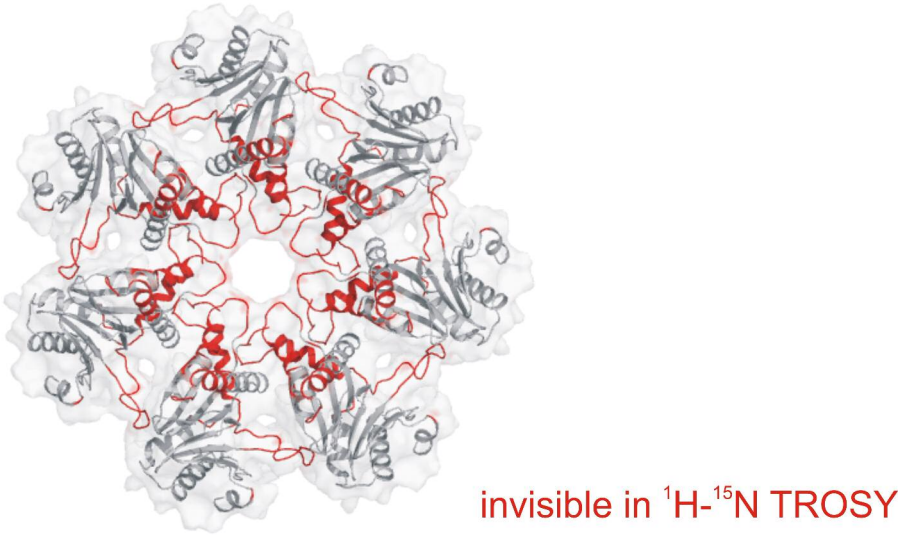

Figure S3. Regions of the single α ring that are not visible in ^{1}H-^{15}N based spectra. The 'invisible' region include residues that undergo ms timescale motions and that can be observed in ^{1}H-^{13}C methyl-TROSY data sets, illustrating the complementarily of ^{15}N and ^{13}C-based experiments.

Table S1. Residues Whose Chemical Shifts are Affected by Chloroquine Binding

^{1}H-^{13}C Methyl TROSY (full proteasome):
Leu 48, Leu 69, Ile 157, Val 217, Ile 223

^{1}H-^{13}C Methyl TROSY (α7α7):
Val 46, Ile 67, Leu 69, Val 101, Leu 106, Val 107, Ile 141, Ile 144, Val 217, Ile 223

^{1}H-^{15}N Amide TROSY (α7):
Ala 92, Arg 93, Ala 96, Gly 104, Ile 109, Leu 112, Gly 140, Asp 142, Gly 145, Cys 151, Ile 157, Gly 166, Tyr 221, Ile 223

Supporting References

1. Sprangers, R. & Kay, L. E. (2007) Quantitative dynamics and binding studies of the 20S proteasome by NMR *Nature.* 445: 618-22.
2. Goto, N. K., Gardner, K. H., Mueller, G. A., Willis, R. C. & Kay, L. E. (1999) A robust and cost-effective method for the production of Val, Leu, Ile (delta 1) methyl-protonated 15N-, 13C-, 2H-labeled proteins *J Biomol NMR.* 13: 369-74.
3. Frank, J., et al. (1996) SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields *J Struct Biol* 116: 190-9.