Supporting Online Material for

Hybrid Molecular Structure of the Giant Protease Tripeptidyl Peptidase II

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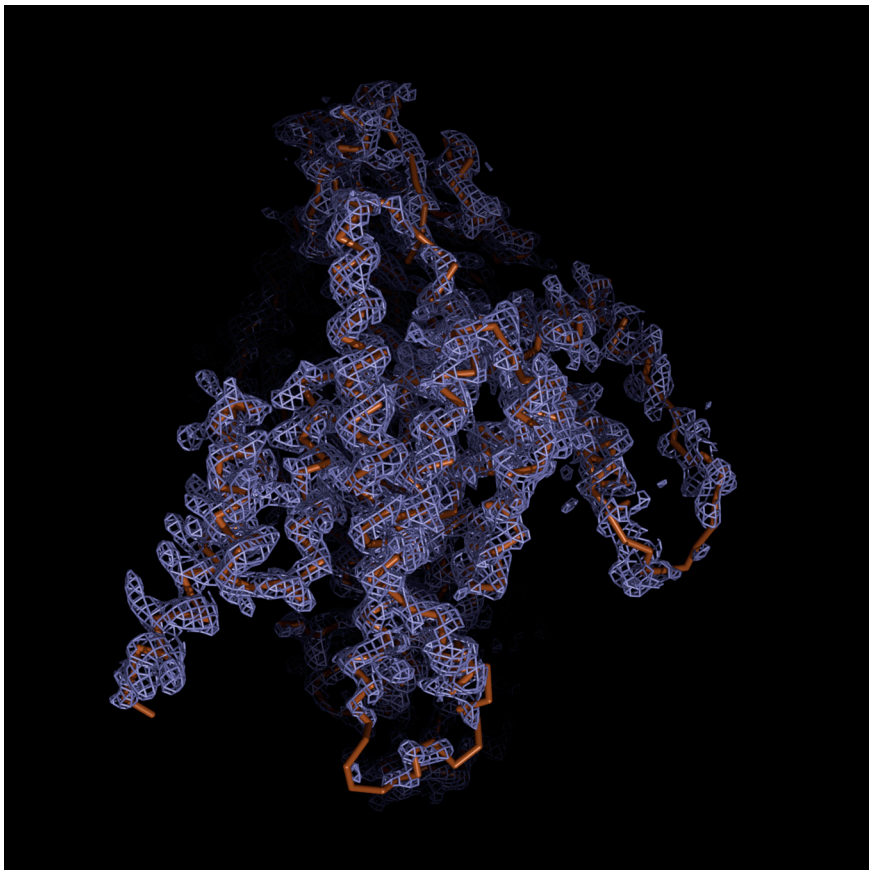
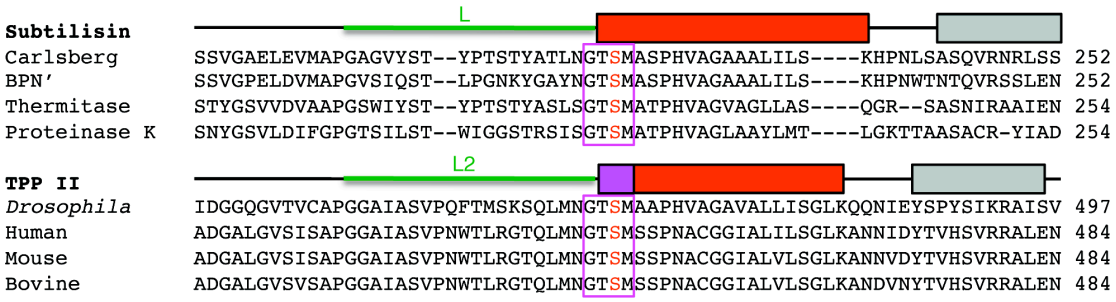
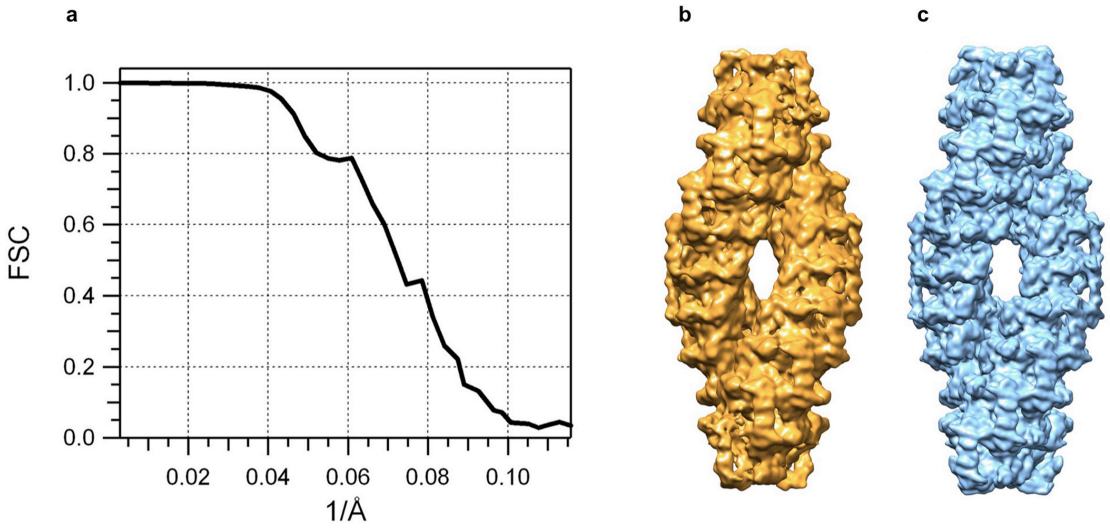


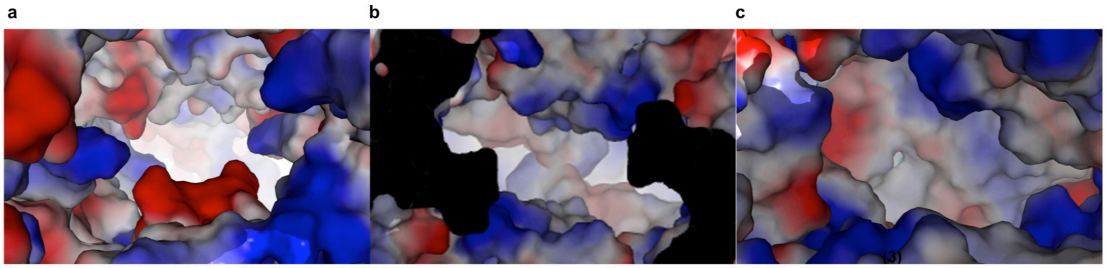
Supplementary Figure 1 Portion of the experimental electron density map. Experimental electron density map (purple) of the TPP II structure, contoured at 1.5 σ, and the α–carbon backbone trace (brown).



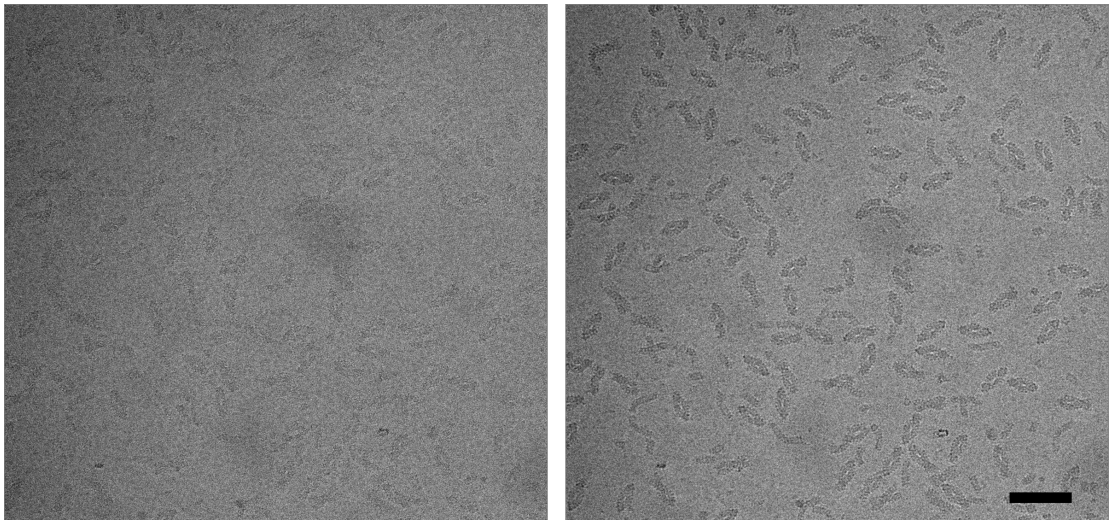
Supplementary Figure 2 Aligned subtilisin and TPP II sequences, where rectangles in red and gray indicate locations of α–helices. The magenta square on the left side of the red rectangle depicts the unwound N–terminal region of the α–helix in the TPP II dimer structure. The amino acid sequence of this region (boxed in purple) is highly conserved in both subtilisin and TPP II families. Active–site serine residues of subtilisin and TPP II family members are shown by red letters. Leading to the active–site serine in *Drosophila* TPP II is the L2 loop (indicated by a green line) and the equivalent L loop in subtilisin Carlsberg (also represented by a green line). Given the sequence homology, it is expected that the unwound helix of TPP II will adopt an α–helical conformation when a hydrogen bond network similar to that in subtilisin is established if the L2 loop is stabilized upon TPP II spindle–assembly. This re–establishment of the hydrogen bond network near the N–terminal end of the α–helix (magenta square) would in turn reposition Ser462, activating the TPP II holocomplex.



Supplementary Figure 3 3D reconstruction of the TPP II spindle by cryo–EM. (**a**) Fourier shell correlation (FSC). (**b**) 3D–reconstruction of TPP II spindles obtained by cryo–EM at 14 Å resolution (determined from the FSC plot at a value of 0.5). (**c**) Pseudo–atomic model of the TPP II holocomplex after low–pass filtering to a resolution of 14 Å is very similar to **b**, indicating the quality of the cryo–EM map and the fit of the X–ray model into the map. The threshold value for isosurface representation was set such that a mass of 6 MDa is included (assuming a density of 1.35 g cm^{-3} for proteins).



Supplementary Figure 4 Cut–away views of entrances to the chambers. Surface potential (red, negative; blue positive) of (**a**) the entrance to the T–joint leading to the antechamber entrance. (**b**) the antechamber entrance (clipped regions of the protein shown in black) and, (**c**) the entrance to the catalytic chamber. Surface potentials were calculated using the vacuum electrostatics surface feature in PyMol.



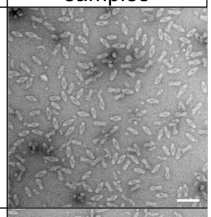
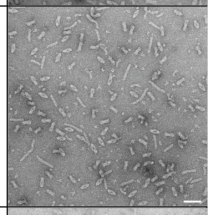
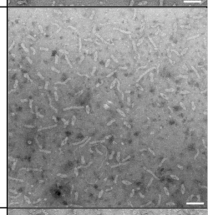
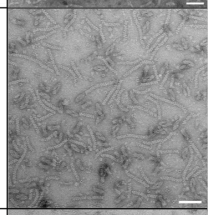
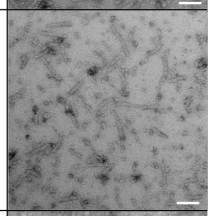
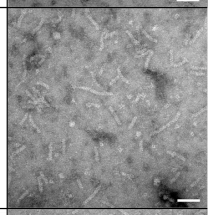
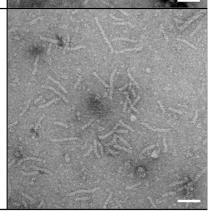
Supplementary Figure 5 Focal pair of vitrified TPP II–particles. Defocus–settings: –1.0 μm (left), –3.0 μm (right). Scale bar: 100nm.

Supplementary Table 1 Kinetic parameters of Glu–mutants determined using a fluorogenic substrate.

	Wild type	E312Q mutant	E343Q mutant
V_{max} / $V_{100/1000}$ (μmol x g^{-1} x min^{-1})	22300	1.85	*5.0/50.2*
K_M (μmol)	410	2700	n.d. (> 10000)
K_{eff} (V_{max} / K_M) (μg^{-1} x min^{-1})	54400	0.69	n.d.

Tripeptidyl peptidase activity was measured using Alanyl–alanyl–phenylalanyl–7–aminomethyl–4–coumarin as substrate. Where V_{max} could not be determined because K_M was too high, the rates obtained at 100/1000 μM substrate are given in italics.

Supplementary Table 2 Mutations of TPP II and effect on activity and assembly (scale bars denote 100 nm).

Mutation	Oligomer state	% of wild-type activity	EM-images of negatively stained TPP II samples
Wild-type	Spindles	100	
E312Q	Single strands and spindles	0.15	
E343Q	Single strands and spindles	0.17	
N374S	Spindles	0.17	
K454E	Single strands	0.16	
L603E/L605E	Single strands	0.18	
R610D	Single strands	0.18	

SUPPLEMENTARY METHODS

Purification of TPP II spindles. Spindles were purified following methods described earlier[17] with modifications. Briefly, TPP II–overexpressing *E. coli* cells suspended in 50 mM potassium phosphate (pH 7.5), 5 mM DTT were disrupted by passage through a cell disruptor device (EmulsiFlex C5, Avestin). The crude lysate was centrifuged at 30,000 g for 15 min. To the supernatant containing TPP II, polyethyleneimine (PEI) was added until no more precipitate was formed while ensuring that the pH did not exceed 9. The treated sample was then centrifuged at 19,000 g for 15 min and the supernatant was subjected to precipitation at 55% (w/v) saturated ammonium sulfate at pH 7.5–8.0. The precipitant was collected by centrifugation at 20,000 g for 20 min, re–suspended in a solution containing 80 mM potassium phosphate (pH 7.5), 5% (v/v) glycerol, 2 mM DTT, and then loaded onto a Superose 6 column (GE Healthcare). Eluted TPP II spindle fractions were further purified on ANX–Sepharose™ (GE Healthcare) that was equilibrated with 40 mM KPO_4, pH 7.8, 2 mM DTT, 5% (v/v) glycerol using a phosphate step gradient (100–400 mM KPO_4, 2 mM DTT, 5% (v/v) glycerol, pH 6.7) for elution. Active fractions were pooled and small aliquots (16 µl) were flash–frozen in liquid nitrogen and stored at liquid nitrogen temperature until grids were prepared for electron microscopy.

TPP II activity assay. Exopeptidase activity measurements of TPP II were carried out as described previously[17]. Briefly, at 30 ºC, the fluorogenic substrate AAF–AMC (Ala–Ala–Phe–7–amino–4–methyl–coumarin) was used at a concentration of either 0.2 mM or 2.0 mM. Reactions were stopped after a period of time ranging from 30 s to 10 min with Tris–HCl, pH 9.5, 1% (w/v) SDS. The release of AMC was measured in a fluorometer calibrated using free AMC.

Reconstruction refinement. Single particle reconstruction refinement was performed using the EMAN–package. The starting model for the refinement was a previous reconstruction, low–pass

filtered to a resolution of 30 Å. The model was refined for 11 iterations with a final angular increment of 2^0. D2–symmetry was imposed during the refinement. The resolution of the final reconstruction was estimated by separating the particle set into two sets and calculating the Fourier shell correlation. According to the 0.5 FSC criterion, the resolution of the reconstruction is ~ 14 Å.

Calculation of protein interfaces. Protein contact surfaces were calculated using the EBI (European Bioinformatics Institute) online server PISA (Protein Interfaces, Surfaces and Assemblies)[43] and a coordinate of the octamer involved in the strand–strand contact.

SUPPLEMENTARY REFERNCES

43. Krissinel, E. & Henrick, K. Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol*. **372**, 774–797 (2007).