Structure *18*

Supplemental Information

Structural and Theoretical Studies Indicate that the Cylindrical Protease ClpP Samples Extended and Compact Conformations

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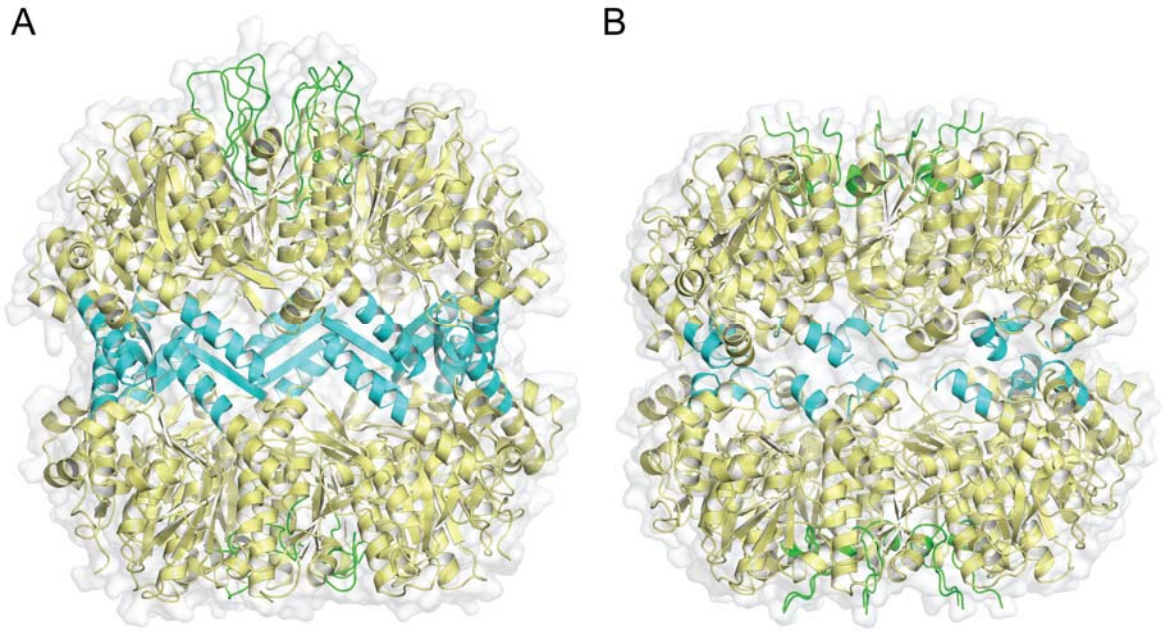

Figure S1. The extended and compact structures of ClpP (related to Fig. 3)
(A) The structure of *E. coli* ClpP (1YG6) showing the ClpP tetradecamer in the extended state (Bewley et al., 2006).
(B) Structure of *P. falciparum* ClpP (2F6I) showing ClpP in the compact state (Vedadi et al., 2007). The handle region is predominantly disordered in *P. falciparum* ClpP, and the two rings are approximately 10 Å closer together. The axial loops in this structure are predominantly disordered, though this is not true of all structures in the compact configuration [e.g. *S. pneumoniae* ClpP (Gribun et al., 2005)].

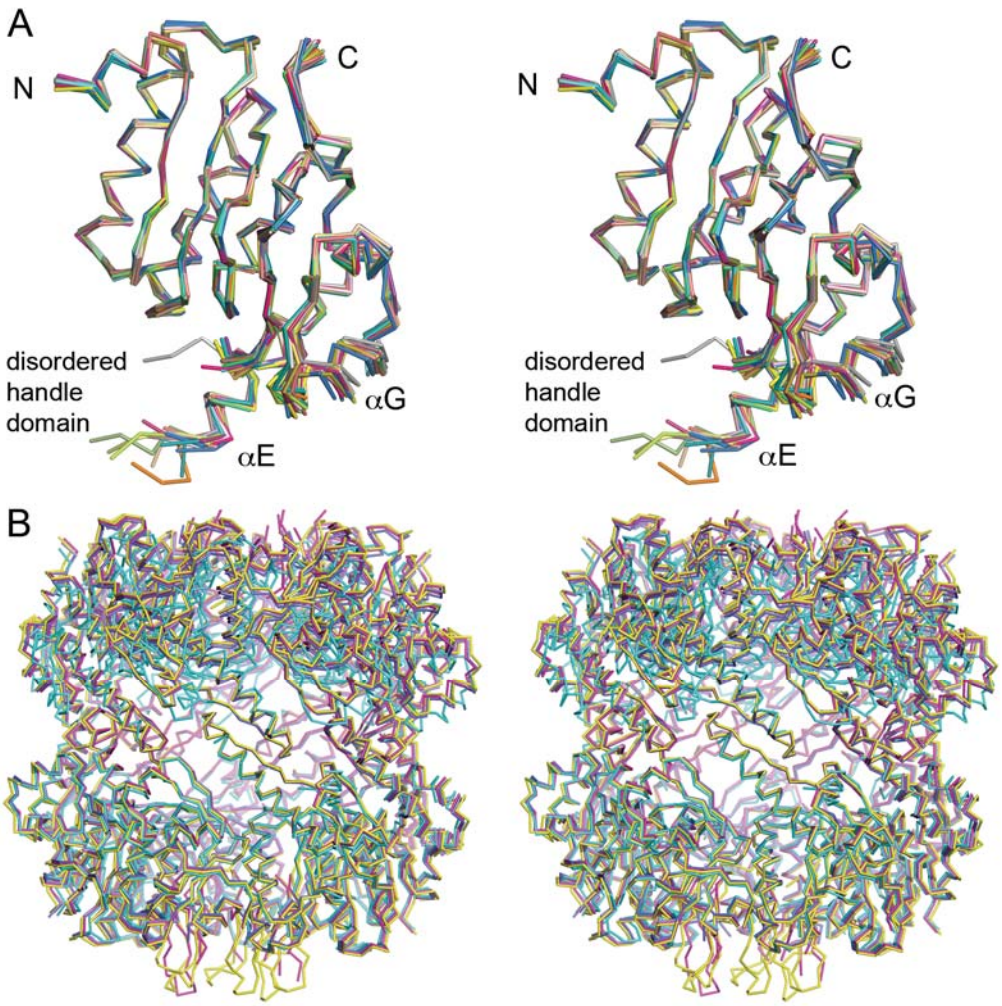

Figure S2. Overlay of *E. coli* ClpP structures (related to Fig. 3)
(A) Overlay of all 28 protomers of the $ClpP^{SS}$ structure. In general, all monomers superpose well except in the region of the handle domain. αG, which interacts with the αG of a second monomer across the ring, also shows some structural variability.
(B) Stereo view of the overlay of the tetradecamer from the four independently determined *E. coli* ClpP structures. $ClpP^{SS}$ is in cyan, 1YG6 is in yellow, 1YG8 is in blue, and 2FZS is in magenta. $ClpP^{SS}$ clearly differs from all of the other structures, which closely resemble one another except in the conformation of the axial loops.

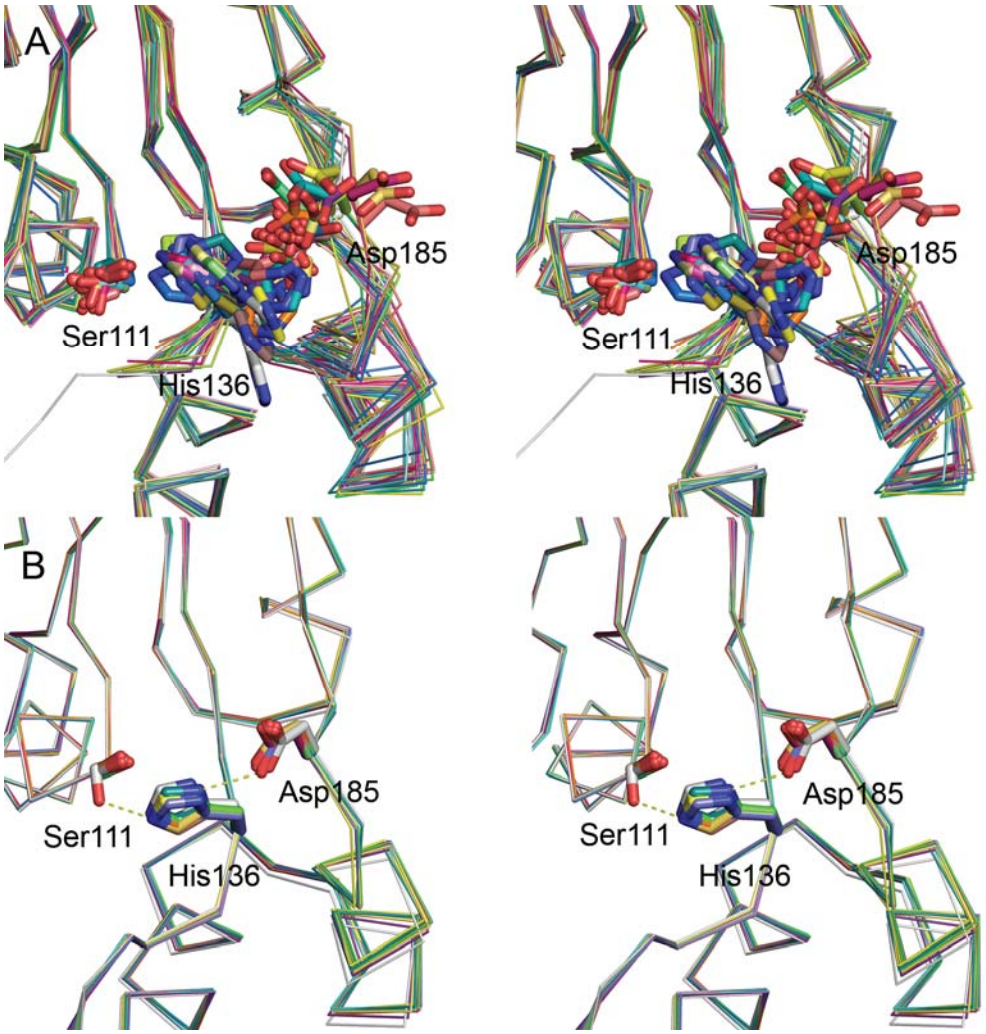

Figure S3. Overlay of the catalytic sites in ClpP (related to Fig. 3)
(A) Stere oview of the overlay of the 28 independent Ser111-His136-Asp185 catalytic triads from the two $ClpP^{SS}$ tetradecamers in the asymmetric unit. The active site shows a high degree of disorder. Ser111 mostly shows differences in rotamer, while His136 and Asp185 show a high degree of variability in both backbone placement and side chain rotamer. None of the individual catalytic sites are properly positioned for catalysis.
(B) Stere oview of the overlay of the 14 independent active sites of WT *E. coli ClpP* (1YG6). The active sites are all very similar, with the exception of Ser111, which samples two preferred rotamers. Aside from the mispositioning of Ser111, these active sites are appropriately pre-organized for catalysis.

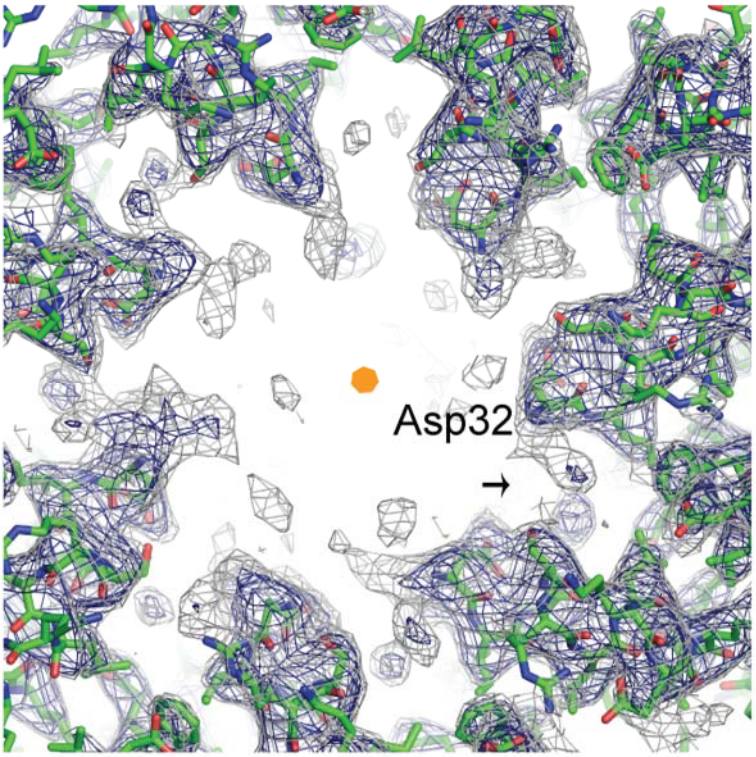

Figure S4. The axial pore region of ClpP^{SS} looking from outside the tetradecamer into the ring (related to Fig. 3)

Mesh shows $2mF_o$-DF_c electron density contoured at 1.0 σ (blue) and 0.5 σ (grey). Even at 0.5 σ, there is no continuous electron density that could correspond to the axial loops, arguing that the axial loops are completely disordered in ClpP^{SS}. All monomers in all of the four axial pores in the asymmetric unit show essentially identical features. The orange heptagon shows the location of the non-crystallographic seven-fold axis. The arrow points to one groove between adjacent helices αA were the N-terminal-most portion of the axial loop would be expected to bind.

Table S1. Disordered residues in the $ClpP^{SS}$ structure (related to Fig. 3)

Tetradecamer 1 (A-N)		Tetradecamer 2 (O-2)	
Chain	Disordered residues	Chain	Disordered residues
A	15-31, 138-151	O	15-31, 139-148
B	15-31, 139-152	P	15-31, 139-151
C	15-31, 138-151	Q	15-31, 138-151
D	15-31, 141-151	R	15-31, 138-150
E	15-31, 138-151	S	15-31, 138-151
F	15-31, 138-147	T	15-31, 138-152
G	15-31, 138-151	U	15-31, 138-151
H	15-31, 138-149	V	15-31, 138-149
I	15-31, 139-151	W	15-31, 138-152
J	15-31, 139-151	X	15-31, 137-151
K	15-31, 139-152	Y	15-31, 138-150
L	15-31, 138-151	Z	15-31, 139-149
M	15-31, 138-151	1	15-31, 138-150
N	15-31, 138-151	2	15-31, 138-151

Supplemental Experimental Procedures

Protein purification

E. coli WT ClpP was purified as previously described (Wojtyra et al., 2003). *E. coli* ClpP(A153C) mutant was generated from WT ClpP using the QuikChange kit (Stratagene) following manufacturer's protocol. The construct was then subcloned into pET9a vector and transformed into SG1146(DE3) Δ*clpP* cells to express untagged ClpP(A153C). A single transformant colony was grown overnight in 10 mL of Luria-Broth (LB) media containing 100 µg/mL ampicillin at 37°C. The overnight culture was inoculated into 1 L LB with 100 µg/mL ampicillin and grown at 37°C. When OD_{600} reached 0.6, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added at a final concentration of 1 mM to induce protein expression for 3 hours at 37°C. Cells were then collected by centrifugation and lysed by French press. Cell debris was removed by centrifugation. The supernatant containing ClpP(A153C) was partially purified by ammonium sulphate precipitation at 30-60% saturation. Precipitated ClpP(A153C) was re-

solubilized and dialyzed in buffer A (50 mM TrisHCl, pH 7.5, 150 mM KCl, 1 mM DTT, and 10% glycerol). The protein was further purified using an anion-exchange column (Q Sepharose). The high salt buffer (buffer B) contained 50 mM TrisHCl, pH 7.5, 1 M KCl, 1 mM DTT, and 10% glycerol. $ClpP^{SS}$ eluted at 250-300 mM KCl. Subsequently, the protein was further purified by size exclusion chromatography using Superdex 200 column equilibrated with buffer A. The protein was then stored in buffer A, but in the absence of DTT. The yield was typically 60 mg of purified $ClpP^{SS}$ from 1 L culture.

Human ClpP was expressed from the plasmid pDT1668 LhclpP, which encodes carboxy-terminal 6xHis tagged human mitochondrial ClpP (a generous gift from Dr. David Dougan, La Trobe University, Australia). pDT1668 LhclpP was transformed into SG1146(DE3) Δ*clpP* strain. The expressed protein was purified on Ni-NTA-agarose (Qiagen) according to manufacturer's protocols.

Protein biochemical characterization

To visualize the mobility of WT ClpP and ClpP(A153C) on reducing and non-reducing SDS-PAGE gels (Fig. 1C), the proteins were incubated for 30 minutes at 37°C in the presence or absence of 10 mM DTT and then incubated with 55 mM iodoacetamide (Sigma) for 30 minutes at 37°C to block free cysteines.

Size exclusion chromatography was carried out using a Superdex 200 HR 10/30 (GE Healthcare) column attached to an AKTA FPLC (GE Healthcare). The column was equilibrated with buffer C (50 mM TrisHCl, pH 7.5, 200 mM KCl) in the presence or absence of 1 mM DTT. Molecular mass standards (Sigma) used were: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66kDa), carbonic anhydrase (21 kDa), and cytochrome c (12.4 kDa). All experiments were performed at 4°C. Proteins were detected using absorbance measured at 280 nm.

CD spectra were measured at 25°C in a 1 mm path length cuvette using a Jasco J-810 spectropolarimeter equipped with a Peltier temperature control device. Prior to the measurement, the protein was incubated at 4°C overnight in buffer C with or without 1 mM DTT. The final protein concentration was 10 μM. Fig. 1D shows the average of three recorded spectra.

Sedimentation velocity analytical ultracentrifugation

Sedimentation velocity experiments were carried out at the Ultracentrifugation Service Facility at the Department of Biochemistry, University of Toronto. Proteins were buffer exchanged into buffer C in the presence or absence of 1 mM DTT using Bio-Spin 6 column (Bio-Rad). Samples were then incubated at 37°C for 2 hours prior to the experiment. Samples were spun at 25,000 rpm (45,500*g* at cell centre) at 20°C in a Beckman Optima Model XL-A analytical ultracentrifuge equipped with An-60 Ti rotor.

The density (ρ) and viscosity (η) of buffer C were calculated to be 1.00909 g/mL and 0.010143 poise at 20°C, respectively, using Sednterp (Laue et al., 1992). Partial specific volumes were calculated to be 0.7387 mL/g for WT ClpP, 0.7382 mL/g for ClpP(A153C), and 0.7407 mL/g for human ClpP using Sednterp based on the amino acid composition of each protein. The sedimentation data were fit to a continuous distribution model c(s) using SEDFIT (Schuck, 2000). The sedimentation coefficients obtained from the fitting were corrected to the density and viscosity of water at 20°C to get $s_{20,w}$.

Peptidase and proteinase assays

The peptidase activity of WT ClpP and ClpP(A153C) under reducing and non-reducing conditions was determined by measuring the rate of cleavage of the fluorogenic peptide N-succinyl-Leu-Tyr-7-amino-4-methyl-coumarin (Suc-LY-AMC) purchased from Sigma. 0.5 μM ClpP was equilibrated in buffer C at 37°C for 2 minutes in the presence or absence of 1 mM

DTT and then 0.1 to 3.0 mM Suc-LY-AMC was added to start the reaction. The fluorescence change resulting from the release of AMC upon cleavage of the Y-AMC bond was monitored for 10 minutes on a Fluorolog 3-222 spectrofluorometer (Jobin Yvon) using an excitation wavelength of 360 nm and an emission wavelength of 440 nm. A standard curve for AMC fluorescence was constructed to convert fluorescence into concentration units in order to determine the amount of AMC generated. K_M and k_{cat} were obtained from double reciprocal plots.

Degradation of casein by ClpAP was carried out by first pre-incubating 1.5 μM of α-casein with 1.2 μM ClpP [WT or ClpP(A153C)], ATP regenerating system (0.32 mg/mL creatine phosphokinase, 16 mM creatine phosphate, and 3 mM ATP), plus or minus 10 mM DTT in buffer D (25 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 300 mM KCl, 0.03% Tween 20, and 10% glycerol) for 3 minute at 37ºC. All concentrations are those of monomers. To start the degradation reaction, 1.0 μM ClpX was added to the mixture incubated at 37ºC. Samples were withdrawn at indicated time points and immediately mixed with 4x Laemmli buffer to stop the degradation reaction. Proteins samples isolated at different degradation time points were separated on SDS-PAGE gels and visualized by Coomassie staining.

The degradation of fluorescein isothiocyanate labeled casein (Sigma) was monitored by fluorescence using a Fluorolog 3-222 spectrophotometer (Horiba Jobin Yvon). Excitation wavelength was set at 490 nm and emission wavelength at 525 nm.

ATPase assays

ATPase Activity of ClpX was measured using the ATP/NADH coupled assay (Norby, 1988). Various concentrations of ClpP or ClpP(A153C), 1.0 μM ClpX , and NADH mixture (0.2 mM NADH, 3.0 mM phosphoenolpyruvate, 0.05 mg/ml pyruvate kinase, 0.025 mg/mL lactate dehydrogenase) were pre-incubated at 37°C for 3 minutes in ClpX buffer (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM KCl, 0.03% Tween 20, and 10% glycerol) plus or minus 10 mM DTT. All concentrations are those of monomers. To start the reaction, 5 mM ATP was added and the change in absorbance at 340 nm was measured for 20 min at 37°C. Each reaction was repeated at least three times. The data were fit to a simple single-site Langmuir binding isotherm.

SUPPLEMENTAL REFERENCES

Bewley, M.C., Graziano, V., Griffin, K., and Flanagan, J.M. (2006). The asymmetry in the mature amino-terminus of ClpP facilitates a local symmetry match in ClpAP and ClpXP complexes. J. Struct. Biol. *153*, 113-128.

Gribun, A., Kimber, M.S., Ching, R., Sprangers, R., Fiebig, K.M., and Houry, W.A. (2005). The ClpP double ring tetradecameric protease exhibits plastic ring-ring interactions, and the N termini of its subunits form flexible loops that are essential for ClpXP and ClpAP complex formation. J. Biol. Chem. *280*, 16185-16196.

Laue, T.M., Shah, B.D., Ridgeway, T.M., and Pelletier, S.L. (1992). In Analytical Ultracentrifugation in Biochemistry and Polymer Science, S.E. Harding, A.J. Rowe, and J.C. Horton, eds. (Cambridge: Royal Society of Chemistry), pp. 90-125.

Merritt, E.A., and Bacon, D.J. (1997). Raster3D: photorealistic molecular graphics. Methods Enzymol. *277*, 505-524.

Norby, J.G. (1988). Coupled assay of Na+,K+-ATPase activity. Methods Enzymol. *156*, 116-119.

Schuck, P. (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. Biophys. J. *78*, 1606-1619.

Vedadi, M., Lew, J., Artz, J., Amani, M., Zhao, Y., Dong, A., Wasney, G.A., Gao, M., Hills, T., Brokx, S.*, et al.* (2007). Genome-scale protein expression and structural biology of Plasmodium falciparum and related Apicomplexan organisms. Mol. Biochem. Parasitol. *151*, 100-110.

Wojtyra, U.A., Thibault, G., Tuite, A., and Houry, W.A. (2003). The N-terminal zinc binding domain of ClpX is a dimerization domain that modulates the chaperone function. J. Biol. Chem. *278*, 48981-48990.