Supplementary information

1. Materials and methods

Protein purification

Synthetic gene coding for human CLPB protein (without first 92 residues corresponding to putative signal peptide), with a 3 aminoacid linker (GSG) linker and TEV protease cleavage site, cloned into pMAL c5x expression vector in NotI/BamHI sites was ordered from GenScript. Vector was transformed into *E. coli* C41 strain, and cultivated in medium [per liter: 18g peptone, 20 g yeast extract, 2.2g KH_2PO_4, 9.4 g KH_2PO_4 0.5% glycerol, 0.1% glucose, 29,2 mg NaCl, 203,3 mG $MgCl_2x6H_2O$, 200 mg ZnCl, 10 mg $CuSO_4$] supplemented witch 100 μg/L ampicillin. The culture was induced at O.D ~1 with 0.5 mM IPTG overnight at 20 ºC. Approx. 20g wet mass of centrifuged cells were suspended in 40 ml buffer (HEPES pH7.8 50 mM, NaCl 1M, 2-mercaptoethanol 20 mM, Triton X-100 0.2%, glycerol 10%) and lysed by French press. From cell lysate clarified by centrifugation (15 min, 95kG) and diluted 1/1 with water, proteins were precipitated with ammonium sulphate at 65% saturation. Dissolved protein pellet was washed twice with buffer (NH_4SO_4 65% saturation, HEPES pH 8.1 20 mM, NaCl 500 mM, 2-mercaptoethanol 5 mM, Triton X-100 0.1%, glycerol 5%) and dissolved in 50 ml of the same buffer without ammonium sulphate. Solution was then applied on 5 mL MBP-Trap column and washed with two column volumes of this buffer. Subsequently, column was washed with 5 column volumes of buffer (HEPES pH 8.1 20 mM, NaCl 100 mM, 2-mercaptoethanol 5 mM, glycerol 5%) and protein was eluted by same buffer supplemented with 25 mM maltose. Fractions containing CLPB proteins were pooled, concentrated by CentriPrep 50 kDa ultrafiltration device to approx. 6 mL, adjusted to 10 mM dithiothreitol and left for 36 h incubation in cold room for proteolytic cleavage of MBP. 0.5 mg TEV protease was added at the beginning of incubation and after overnight incubation second portion of 0.5 mg was added. The cleaved MBP and TEV protease were removed on Superdex200 pg 16x600 gel filtration column. The CLPB containing fractions were pooled and protein concentration was determined to equal 22.5 μM by measuring differential absorbance spectrum in 6M urea pH 12.5 and 7.1 according to [Suppl. Literature 1]

Other proteins used in this study were taken from collection of Molecular and Cellular Biology Department, Intercollegiate Faculty of Biotechnology, university of Gdansk

ATPase assay

ATPase assays were performed in enzyme-coupled lactate dehydrogenase/pyruvate kinase spectrometric assay essentially as described by Norby [Suppl. Literature 2]. Decrease of NADH absorbance at 340 nM was measured in a sample containing protein with indicated amounts of ATP diluted with buffer to final concentration of: 0,2 mM NADH, 1 mM phosphoenolpyruvate, 2,5%

glycerol, 5 mM HEPES pH 7.8, 100 mM NaCl, 50 mM KCl, 40 mM $MgCl_2$, 2.5 mM βME in final volume of 120 µL containing 0,2 µl LDH/PK mixture (17 U/ml) (Sigma).
For Michaelis-Menten plot, reactions were measured in 10 mm pathlength cuvette in Jasco V-650 spectrofluorimeter and ATP turnover was calculated as described. Data was analyzed in GraphPrism3 software by fitting to Michaelis-Menten model for Km and Vmax determination for at least three independent reactions.
For gel filtration fractions ATPase activity on Fig. 1, absorbance change was recorded with Beckman Coulter Plate Reader DTX 880 and slopes of linear portions of the curves were normalized relative to the fastest reaction (=1).

Luciferase refolding assay

Firefly luciferase (40 µM) was diluted in buffer (HEPES-KOH pH 7.5 25 mM, KCl 75 mM, $MgCl_2$ 15 mM, urea 6M). Further dilution was performer in buffer (HEPES-KOH pH 7.5 25 mM, KCl 75 mM, $MgCl_2$ 15 mM) to the final concentration of 0,5 µM. It was refolded at a concentration of 0,1 µM in the presence of: Ydj1 (1 µM), Ssa1 (1 µM), Hsp104 (1 µM), human CLPB (1 µM) in respective combinations or alone as indicated in buffer (HEPES-KOH pH 7.5 25 mM, KCl 75 mM, $MgCl_2$ 15 mM). Some of the reactions were supplemented with ATP regeneration system (20 mM creatine phosphate, 0,1 µg creatine kinase) as indicated. Upon protein refolding activity increasing luciferase activity was measured. The measurement was taken after every 60 minutes of incubation to the final incubation time of 240 min (4h) using Luciferase Assay Kit (Promega) with Sirius Luminometer (Berthold). The experiments were repeated twice.

GFP refolding assay

GFP (111,1 µM) was incubated for 15 minutes in 85^0C in buffer (Tris-HCl pH 7.5 50 mM, NaCl 150 mM, 2-mercaptoethanol 5 mM, glycerol 10%, dithiothreitol 84 mM). It was refolded at a concentration of 1,1 µM in the presence of Ydj1 (1 µM), Ssa1 (1 µM), Hsp104 (1 µM), human CLPB (1 µM) in respective combinations or alone as indicated in buffer (HEPES-KOH pH 8.0 28 mM, potassium glutamate 60 mM, Mg acetate 14 mM, dithiothreitol 7 mM, glycerol 10%, ATP 10 mM) . Upon protein renaturation activity increasing fluorescence signal was measured using Beckman Coulter Plate Reader DTX 880.

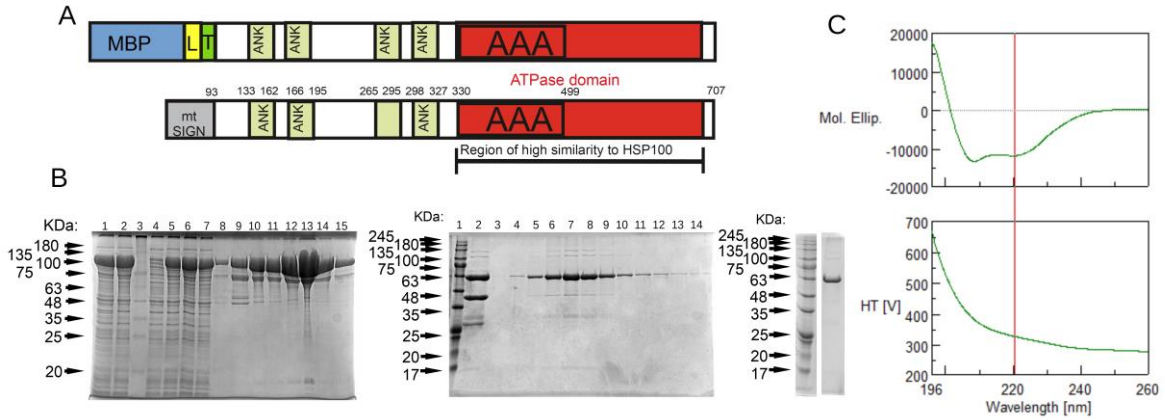
Molecular imaging

Atomic force microscopy of 200nM CLPB protein, was performed in 20 mM HEPES buffer pH 7.8, 100 mM KCl and 5 mM ATP at room temperature in PeakForce Tapping mode using BioScope Resolve AFM (Bruker). The ScanAsyst-Fluid+ probe (Bruker) was used for protein imaging (resonant frequency f_0 = 150 kHz; spring constant k = 0,7 N/m).

Image was taken at 256 × 256 pixels with PeakForce Tapping frequency of 1.5 kHz and amplitude 100 nm. Height sensor signal was used to display the protein image using NanoScope Analysis v1.9.

Electron microphotography of uranyl acetate negatively stained samples of CLPB protein were performed by Laboratory of Electron Microscopy, Faculty of Biology, University of Gdansk.

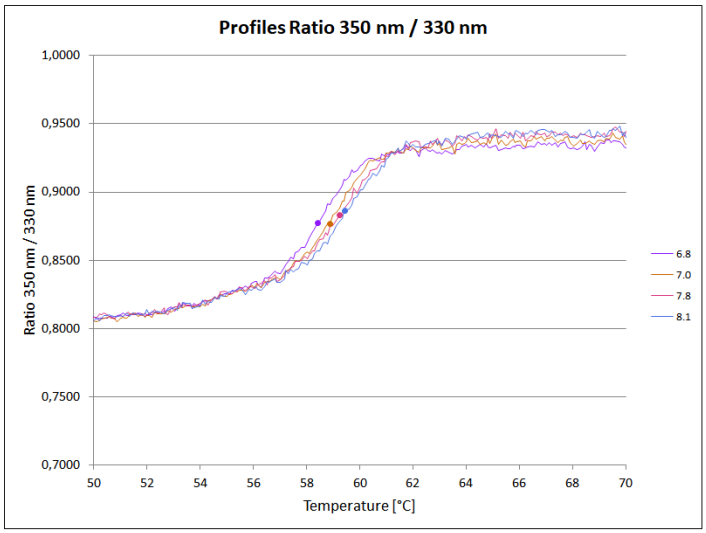
Supp. Fig. 1



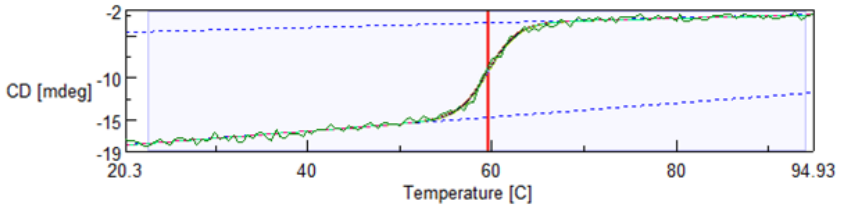
A. Top, expressed construct for CLPB purification. Maltose binding protein domain (MBP) together with a linker sequence (NSSSNNNNNNNNNLGIEGRISHMSMGGR) containing FactorXa cleavage site (not used) originated from the pMal-c5x vector. The synthetic construct sequence involved a short linker (GSG) and TEV recognition sequence (ENLYFQGP) which preceded the 93-707 sequence of CLPB protein and a stop codon.
   Below, domain structure of CLPB protein. Marked are: mitochondrial import signal peptide (putative end position at residue 92), ankyrin motifs as marked by UniProt entry Q9H078, ATPase domain with AAA+ module, marked as a region of high similarity to Hsp100 disaggregases.
B. Purification of human CLPB protein. First gel, lanes: 1) clarified lysate, 2) ammonium sulphate precipitate, 3) molecular weight marker, 4-7) flow-through from MBP-Trap column, 8-15) peak elution fractions. Second gel, 1) MW marker, 2) TEV digestion mixture, 3-15) Superdex 200 pg 16x600 gel filtration fractions after TEV cleavage. Third gel, MW marker, 1 µg of final purified CLPB preparation. The size markers used were EurX Perfect Tricolor Protein Ladder.
C. CD spectrum of human CLPB protein. CLPB was diluted 8-fold with 10 mM KPi buffer pH 7.8 and measured as described above. Red line, 220.4 nm maximum used in subsequent melting curve measurement. Upper plot, CD spectrum, lower plot, photomultiplier voltage.

Sup. Fig. 2

A



B



CLPB protein stability

A). Thermal stability scans at indicated pH values in NanoTemper Tycho NT.6 microcapillary fluorescence device. Ratio of intensities pf intrinsic protein fluorescence emitted at 350 and 320 nm is plotted against heating temperature, revealing unfolding changes in tertiary structure. Dots represent deflection points.

B) Melting curve of circular dichroism signal at 220.4 nm, measurement conditions as in supp. Fig. 1 B.

Supplementary references

[1] N. Greenfield, Using circular dichroism spectra to estimate protein secondary structure. Nat. Protocols1(6) (2006):2876-2890. doi:10.1038/nprot.2006.202.

[2] J. Norby, Coupled assay of Na+, K+ -ATPase activity, Methods in enzymology 156 (1988):116-119