Supporting Information for:

Active-site directed chemical tools for profiling mitochondrial Lon protease

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Materials. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. All cloning reagents were purchased from Promega, New England Biolabs, Invitrogen and USB Corporation. HeLa cells were purchased from ATCC. Cell culture media and reagents were purchased from MediaTech, USA Scientific, and Invitrogen. Tris, IPTG, chromatography media, DTT, $Mg(OAc)_2$, trypsin, kanamycin, chloramphenicol, ATP, dansyl chloride, DMSO, Tween 20, and all other materials were purchased from Fisher, Sigma, and Amresco.

Synthesis of peptide substrates and inhibitor. Synthesis of FRETN 89-98, FRETN 89-98Abu, and DBN93 (**Figure 1**) were performed as previously described (*1, 2*).

Expression and purification of recombinant human Lon. Human Lon was expressed and purified as previously described (*3*).

Cloning of recombinant ClpX and ClpP. Using a modified method of Kang et al (*4*), the ClpP gene was amplified from genomic DNA using oligonucleotides oJH075 (5'-GACCCGGCATATGCCGCTCATTCCCATCG-3') and oJH114 (5'-GGCCCAGCTCGAGGGTGCTAGCTG-3'). The ClpX gene was amplified from genomic DNA using oligonucletoides oJH073 (5'-CACCAGCACATATGGCCTCAAAAGATG G-3') and oJH115 (5'-GCAATATGACCTCGAGGCTGTTTGCAG-3'). The resultant PCR products were cloned into the NdeI and XhoI sites of pET24c and transformed into XL1Blue cell line for sequencing confirmation. The ClpX plasmid contained a mutation E373K which was changed back to wild-type using Quick-Change kit from Qiagen with

primers oJH125 (5'-CATCAGCAGGAGGAAAAATGAAAAGTATCTTGGATTTGGAACACC -3') and oJH126 (5'- GGTGTTCCAAATCCAAGATACTTTTCATTTTTCCTCCTGCTGATG -3'). Following sequence confirmation, the plasmids ClpP and ClpX were transformed into Rosetta (DE3).

Expression and purification of recombinant ClpX and ClpP. Recombinant wild type His-tagged human ClpX and human ClpP were overexpressed in Rosetta (DE3) using the plasmids ClpX and ClpP, respectively. Cells were grown in superbroth (SB) containing 30µg/mL kanamycin and 34 µg/mL chloramphenicol at 37 ℃ to OD_{600} ~0.7. Cultures were then induced with 0.5 mM IPTG for 21 hours at 25 ℃. Cells were harvested and pellets were resuspended in loading buffer containing 50 mM Tris (pH 8.1), 150 mM NaCl, 10 mM imidazole, 15% glycerol and 0.05% Tween 20, then lysed by homogenation and sonication. Lysate was applied to Ni-NTA column equilibrated in loading buffer. The column was washed with 5 column volumes of loading buffer, with flow-through collected. The column was then washed with 8 volumes of wash buffer containing 50 mM Tris (pH 8.1), 500 mM NaCl, 10 mM imidazole, 15% glycerol and 0.05% Tween 20, with the flow-through collected. The column was eluted with 5 column volumes elution buffer containing 50 mM Tris (pH 8.1), 300 mM NaCl, 200 mM imidazole, 15% glycerol and 0.05% Tween 20, with 1 mL fractions collected. Collected fractions were analyzed on 12.5% SDS-PAGE gels stained by Coomassie. Fractions containing purified protein were pooled and concentrations were determined by

Bradford assay (*5*), using BSA as a standard. Protein was then aliquoted and stored at -80 ℃.

Time dependent inhibition of peptide cleavage by DBN93. Fluorescent peptidase assays were performed on a Fluoro-Max 3 spectrophotometer (Horiba group). All reactions contained 50 mM Tris (pH 8.1), 5 mM $Mg(OAc)_2$, 150 mM NaCl, 2 mM DTT, 1 mM FRETN 89-98 and 150 nM human Lon. After 1 min at 37 ℃, 1 mM ATP was added. After an additional 90 seconds, varying amounts of DBN93 were added and fluorescent emission at 420nm was monitored for 2400 s with excitation at 320 nm. A mixture of 10% fluorescent/90% non-fluorescent peptide substrate was used to avoid complications arising from the inner filter effect (*35*). Kinetic parameters of inhibition of human Lon by DBN93 were derived using methods previously used for S. Typhimurium Lon (*23, 25*). For reactions containing no inhibitor, steady state velocities were determined from the linear phase of the reaction time course using Kaleidagraph (Synergy). For reactions containing inhibitor, the steady state velocities were determined by fitting the reaction time course (as in manuscript **Figure 3 panel a)** using Kaleidagraph with equation 1

$$P = v_{ss}t + \frac{v_i - v_{ss}}{k_{\text{int } er}}\left[1 - e^{-k_{\text{inter}}t}\right] \qquad \text{Equation 1}$$

where P is the amount of peptide cleaved, determined by converting fluorescent signal to amount of peptide cleaved using the fluorescence upon complete digestion with trypsin, v_{ss} is the final steady-state rate, t is time, v_i is the initial rate, and k_{inter} is the rate constant for the interconversion of v_{ss} and v_i.

Apparent K_i values were determined by fitting v/v_0 vs [I] to equations 2 and 3 (**Figure 3, panel b**), where E is enzyme concentration and I is inhibitor concentration.

$$\frac{v_i}{v_0} = 1 - \frac{\left([E]+[I]+K_i^{app}\right) - \sqrt{\left([E]+[I]+K_i^{app}\right)^2 - 4[E][I]}}{2[E]} \qquad \text{Equation 2}$$

$$\frac{v_{ss}}{v_0} = 1 - \frac{\left([E]+[I]+K_i^{*app}\right) - \sqrt{\left([E]+[I]+K_i^{*app}\right)^2 - 4[E][I]}}{2[E]} \qquad \text{Equation 3}$$

Apparent K_i and K_i^* values from experimental data were converted to true K_i and K_i^* values by equation 4, where S is substrate concentration and K_m is the reported K_m value for the peptide (*27*).

$$K_i^{app} = K_i\left(1 + \frac{[S]}{K_m}\right) \qquad \text{Equation 4}$$

Using this analysis the following kinetic parameters were determined: K_i (μM) = 1.35 ± 0.19 and K_i^* (μM) = 0.014 ± 0.001. These values show close agreement to those derived from global fitting of the experimental data to a two-step mechanism of inhibition as shown in **Table 1** of the manuscript.

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