Supporting Information

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SI Methods

GFP-15 Unfolding Assay. Reaction mixtures (100 μL) contained buffer A [20 mM Tris·HCl, pH 7.5, 100 mM KCl, 5 mM DTT, 0.1 mM EDTA, and 10% glycerol (vol/vol)], 0.005% Triton X-100 (vol/vol), 4 mM ATP or 2 mM ATP, and 2 mM ATPγS (Roche) where indicated, an ATP regenerating system (20 mM creatine phosphate and 6 μg creatine kinase), 10 mM $MgCl_2$, 2.0 μM GFP-15, 3.0 μM $GroEL_{trap}$, and 1.0 μM total ClpB. GFP-15 contains the N-terminal 15 aa of the P1 RepA replication initiator protein fused to the C-terminus of GFP (1). A mutant form of GroEL, $GroEL_{trap}$, that binds but does not release unfolded proteins was included in the reaction mixtures to prevent GFP-15 refolding (2). Unfolding was monitored over time at 23 °C using a Varian Cary Eclipse fluorescence spectrophotometer with a well-plate reader. Excitation and emission wavelengths were 395 and 510 nm, respectively. For subunit mixing experiments, $ClpB_{(wt)}$ and ClpB mutant proteins, as indicated, were added to a total ClpB concentration of 1.0 μM and reactions initiated by the addition of MgATP. GFP-15 unfolding rates were determined from the initial linear decrease in fluorescence intensities.

GFP Reactivation. GFP reactivation assays (100 μL) contained buffer A, 4 mM ATP, an ATP regenerating system as above, 10 mM $MgCl_2$, 10 μL heat-aggregated GFP (heated 10 min at 80 °C at 4.5 μM), and 1.0 μM total ClpB, either $ClpB_{(wt)}$, ClpB mutant, or a mixture containing both in various ratios as indicated. Reactions were initiated by the addition of MgATP, and reactivation was monitored over time at 23 °C as described above for GFP-15 unfolding. GFP reactivation rates were determined from the initial linear increase in fluorescence intensities.

MDH Disaggregation. Reaction mixtures (60 μL) contained buffer A, 5 mM ATP, an ATP regenerating system as above, 0.005% Triton X-100 (vol/vol), 17 mM $MgCl_2$, 25 μL heat-aggregated [^{3}H]MDH (prepared by heating 2.0 μM [^{3}H]MDH in 50 mM Tris·HCl, pH 7.5, 150 mM KCl, 2 mM DTT, and 0.1 mM EDTA for 33 min at 47 °C), 1.4 μM DnaK, 0.25 μM DnaJ, and 0.13 μM GrpE, and 0.5 μM total ClpB, either $ClpB_{(wt)}$, ClpB mutant, or a mixture containing both in various ratios as indicated. Reactions were initiated by the addition of MgATP. After a 40-min incubation at 30 °C, reactions were stopped with 75 mM EDTA and 75 mM $(NH_4)_2SO_4$. The soluble fraction of [^{3}H]MDH was separated by centrifugation and quantified by scintillation counting.

GFP-38 Reactivation. Reactivation assays (100 μL) contained buffer A, 4 mM ATP, an ATP regenerating system as above, 10 mM $MgCl_2$, 5 μL heat-aggregated GFP-38 (heated 15 min at 80 °C at 14 μM, frozen on dry ice, thawed, and used immediately), 0.9 μM DnaK, 0.15 μM DnaJ, 0.08 μM GrpE, and 0.5 μM total ClpB, either $ClpB_{(wt)}$, ClpB mutant, or a mixture containing both in various ratios as indicated. Reactions were initiated by the addition of MgATP, and reactivation was monitored over time at 23 °C as described above for GFP-15 unfolding. GFP-38 reactivation rates were determined from the initial linear increase in fluorescence intensities.

RepA Activation. Reaction mixtures (20 μL) contained buffer A, 4 mM ATP, 10 mM $MgCl_2$, 50 μg/mL BSA, 0.005% Triton X-100 (vol/vol), 4 nM RepA, and 1.0 μM total ClpB, either $ClpB_{(wt)}$, $ClpB_{(B1,B2)}$, or a mixture containing both in various ratios as indicated. Reactions were initiated by the addition of MgATP. After 10 min at 23 °C, 25 mM EDTA was added, and the reactions were chilled to 0 °C. Calf thymus DNA (1 μg) and 15 fmol of [^{3}H]*ori*P1 plasmid DNA were added. After 5 min at 0 °C, the mixtures were filtered through nitrocellulose filters, and retained radioactivity was measured.

ATPase. Reaction mixtures (50 μL) contained buffer A, 0.005% Triton X-100 (vol/vol), 4 mM ATP or 2 mM ATP, and 2 mM ATPγS as indicated, 0.1 μCi of [γ-^{33}P]ATP (>3,000 Ci/mM; Perkin Elmer), 10 mM $MgCl_2$, and 1.0 μM total ClpB, either $ClpB_{(wt)}$, $ClpB_{(B1,B2)}$, or a mixture containing both in various ratios as indicated. Reactions were initiated by the addition of MgATP, incubated 30 min at 25 °C, and analyzed as described in ref. 3.

1. Hoskins JR, Yanagihara K, Mizuuchi K, Wickner S (2002) ClpAP and ClpXP degrade proteins with tags located in the interior of the primary sequence. *Proc Natl Acad Sci USA* 99:11037–11042.
2. Weber-BanEU, Reid BG, Miranker AD, Horwich AL (1999) Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. *Nature* 401:90–93.
3. Shacter E (1984) Organic extraction of Pi with isobutanol/toluene. *Anal Biochem* 138:416–420.