Figures and figure supplements

Interactions between a subset of substrate side chains and AAA+ motor pore loops determine grip during protein unfolding

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Figure 1. Effects of cassette sequence on GFP unfolding and degradation. (A) Starting at the N terminus, substrates contained residues 1–229 of A. victoria GFP (PDB 1GFL, Yang et al., 1996), a cassette with 12 variable residues, and a partial ssrA degron. (B) Method for measuring intracellular degradation of substrates by ClpXΔN/ClpP. (C) Cellular fluorescence depends upon ClpXΔN/ClpP expression and cassette sequence (listed in Table 1). (D) Fraction intracellular degradation for substrates bearing different cassettes. (E) Fits of the substrate dependence of degradation in vitro to a hyperbolic Michaelis-Menten equation. (F) $V_{max}$ values for different substrates. In panels, C–F, values represent averages (± S.D.) of three biological replicates.

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Figure 2. A small subset of tail residues mediate grip during GFP unfolding. (A) Fraction intracellular degradation for substrates with tails containing LYV tripeptides in otherwise all-glycine cassettes. Gly12 and GA substrates were included as internal controls. (B) Fraction intracellular degradation for substrates with tails containing one tyrosine (Y) in otherwise all-glycine cassettes. Gly12 and GA substrates were included as internal controls. (C) V_{max} values from Michaelis-Menten analysis of degradation of purified substrates with single-tyrosine cassettes. (D) Rates of ATP hydrolysis by ClpX^{DN} (0.1 μM hexamer) in the presence of ClpP (0.3 μM 14-mer) in the absence (-) or presence of different substrates (15 μM monomer). (E) ATP cost of degrading substrates with single-tyrosine cassettes. Note that the Y-axis is logarithmic. In all panels, values represent averages (± S.D.) of three biological replicates.

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Figure 2—figure supplement 1. Comparison of $K_M$ values for substrates tested in vitro; comparison of fitted values for $K_M$ for substrate degradation. Values are the average of three biological replicates ± S.D. None of the substrates exhibited a substantial increase in $K_M$, indicating that differences in degradation rates result from differences in grip rather than in initial substrate recognition.
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Figure 2—figure supplement 2. Stimulation of ClpXP ATP hydrolysis by purified substrates. (A) Rates of ATP hydrolysis by ClpX<sup>DN</sup> (0.1 μM hexamer) in the presence of ClpP (0.3 μM 14-mer) in the absence (–) or presence of different substrates (15 μM monomer). (B) ATP cost of degrading substrates. In both panels, values represent averages (± S.D.) of three biological replicates.

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Figure 3. Side-chain grip effects at tail-position 4. (A) In substrates with otherwise all-glycine cassettes, fraction intracellular degradation depends on side-chain identity at tail-position 4. (B) Comparison of degradation in vivo for substrates with Thr or Val at tail-position four or Glu or Gln at tail-position 4 (Student’s two-tailed t-test significance; Val/Thr: t = 6.37, df = 4; Glu/Gln: t = 5.47, df = 4). (C) \(V_{\text{max}}\) values from Michaelis-Menten analysis of degradation of purified substrates. (D) Effects of position-4 residues, color-coded by side-chain properties, on \(V_{\text{max}}\). (E) Comparison of degradation in vitro between substrates with Ala, Ser, Cys, Thr, or Val at tail-position four or Glu or Gln at tail-position 4 (Student’s two-tailed t-test significance; Val/Thr: t = 13.3, df = 4; Glu/Gln: t = 5.49, df = 4). (F) ATP cost of degrading substrates with Ala, Cys, Thr, Val, Glu, or Gln at tail-position 4. With the exception of panel A, where Gly\(_{12}\) and GA values represent averages (± S.D.) of nine biological replicates, all values represent three biological replicates.

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**Figure 4.** Multiple substrate residues contribute synergistically to grip. (A) GA and Ala-4 cassette sequences. A heatmap of $V_{\text{max}}$ values from Figure 2C is overlaid to show contribution of single tyrosine residues as each tail position. (B) Fraction intracellular degradation of substrates with one alanine at tail-position 4 and a second alanine at a variable position in otherwise all-glycine cassettes. (C) Comparison of intracellular degradation for a subset of substrates, including Ala-1. (D) $V_{\text{max}}$ values from Michaelis-Menten analysis of degradation of purified substrates. (E and F) Michaelis-Menten $V_{\text{max}}$ values for purified substrates with one tyrosine (E) or valine (F) at tail-position four and a second tyrosine (E) or valine (F) at each tail position in otherwise all-glycine cassettes. Overlaid dashed lines indicate degradation rate for the parental Tyr-4 (E) or Val-4 (F) substrates. In all panels, values represent averages ($\pm$ S.D.) of three biological replicates.

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Figure 4—figure supplement 1. Degradation of Dual-Tyr substrates centered at tail position 3. $V_{\text{max}}$ values for degradation from Michaelis-Menten analysis of purified substrates with one tyrosine at tail-position three and a second tyrosine at a variable position in otherwise all-glycine cassettes. Relative degradation for substrate tails with a single Tyr residue at position 3 or 4 indicated by dashed lines. Values represent averages ($\pm$ S.D.) of three biological replicates.

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Figure 5. Only a subset of pore-1 loops in ClpX appear to mediate substrate grip. (A) Model of an extended poly-alanine substrate in the axial pore of ClpX and its interactions with different pore-1 loops based on cryo-EM structures of ClpX (X.Fei, T.A. Bell, B.M. Stinson, S. Jenni, T.A. Baker, S.C. Harrison, and R.T. Sauer, in preparation). Similar loop-substrate interactions are observed in the yeast AAA+ protease Yme1 (Puchades et al., 2017). On the right, a heatmap of $V_{\text{max}}$ values from Figure 2C is shown. The substrate tail residues are numbered relative to where a folded domain would be expected to sit at the apical surface of the AAA+ ring during unfolding. Tail residues 2–6, which promote strong grip in ClpX, are positioned to interact with the three pore-1 loops at the top of the axial pore. (B) Two models for asymmetric contribution of pore-1 loops to substrate grip.

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