Pore loops of the AAA+ ClpX machine grip substrates to drive translocation and unfolding

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Abstract

Proteolytic AAA+ unfoldases use ATP hydrolysis to power conformational changes that mechanically denature protein substrates and then translocate the polypeptide through a narrow pore into a degradation chamber. We show that a tyrosine in a pore loop of the hexameric ClpX unfoldase links ATP hydrolysis to mechanical work by gripping substrates during unfolding and translocation. Removal of the aromatic ring in even a few ClpX subunits results in slippage, frequent failure to denature substrate, and an enormous increase in the energetic cost of substrate unfolding. The tyrosine is part of a conserved aromatic-hydrophobic motif, and the effects of mutations in both residues vary with the nucleotide state of the resident subunit, supporting a model in which nucleotide-dependent conformational changes in these pore loops drive substrate translocation and unfolding, with the aromatic ring transmitting force to the polypeptide substrate.

Introduction

Specialized AAA+ molecular machines harness the energy of ATP hydrolysis to power protein unfolding and multimer disassembly in all cells[1,2]. Such machines, typically active as ring hexamers, initiate unfolding by pulling an exposed peptide tag through a narrow axial pore and ultimately translocate the unfolded polypeptide into the degradation chambers of proteases like ClpXP, ClpAP, HslUV, FtsH, Lon, and the proteasome[3–5] (Fig. 1a). How, during a power stroke, are nucleotide-dependent changes in the conformation of a AAA+ machine transferred to the polypeptide substrate to drive translocation and unfolding? Answering this question is essential for understanding how these molecular wrecking machines function.

In almost all AAA+ unfoldases, a loop with a conserved aromatic-hydrophobic (Ar-Φ) dipeptide protrudes from every subunit into the central pore (Fig. 1b)[6,7]. Ar-Φ loop mutations have been shown to eliminate or reduce the activity of numerous AAA+

proteases, and it is commonly assumed that these loops play critical roles in translocation and unfolding by transmitting force to substrates[7–17]. However, Ar-Φ loops have also been implicated in the initial binding of substrates and in controlling rates of ATP hydrolysis, and defects in either of these processes could also account for the mutant phenotypes observed. An analogy with a macroscopic machine is apt. Imagine trying to determine how an automobile functions by altering various parts. The car can be disabled or severely slowed by obstructing the flow of fuel to the engine, but it would be incorrect to conclude that the fuel pump or carburetor transmits force from the motor to the wheels.

ClpXP is a AAA+ protease that consists of the ClpX ATPase and the ClpP peptidase[5]. Here, we characterize the effects of Ar-Φ loop mutations in ClpX from *E. coli*. By saturating substrate binding and correcting for altered rates of ATP hydrolysis, we demonstrate specific roles of the Ar-Φ loops in gripping, translocating, and unfolding substrates. Moreover, we show that these loop activities vary as individual ClpX subunits assume ATP-bound, ATP-hydrolyzing, or nucleotide-free states. These results strongly support a model in which nucleotide-dependent conformational changes in the Ar-Φ loops drive substrate translocation and unfolding, with the aromatic ring transmitting force to the polypeptide substrate.

Results

System and experimental logic

In this work, we use variants of *E. coli* ClpX with covalently linked subunits to examine Ar-Φ loop function during proteolysis of native or denatured substrates bearing the ssrA-degradation tag. This system has several important features. ClpP degradation provides a readout of ClpX activity. For example, ClpXP degradation of the folded titin-ssrA protein requires recognition, translocation, and robust unfolding by ClpX, whereas degradation of titin^{CM}-ssrA, a variant permanently unfolded by carboxymethylation, requires only recognition and translocation[18]. Covalent linkage of ClpX subunits also allows loop mutations to be introduced into just a few subunits of the hexamer, thereby preventing severe loss of function. Because these single-chain ClpX mutants retain activity, we can determine kinetic parameters that distinguish substrate-binding defects from rate-limiting translocation/unfolding defects and also can establish whether activity changes are caused by alterations in the coupling between ATP hydrolysis and mechanical work.

Moreover, the use of covalently linked ClpX variants allows the importance of nucleotide state to be probed. ClpX and related hexameric ATPases function by asymmetric mechanisms in which individual subunits assume ATP-bound, ATP-hydrolyzing, or nucleotide-free empty states during the ATPase cycle[19–23]. By using mutations in subsets of linked ClpX subunits, we mimic asymmetry in the wild-type enzyme and can probe the relationship between the nucleotide state of a subunit and the role of its pore loop in ClpX function. For example, RWE/RWE ClpX is a dimer of covalent trimers, each containing an ATPase-defective subunit in a permanent empty-state conformation (R subunit; R370K mutation), a wild-type subunit (W subunit), and a hydrolysis-defective ATP-state subunit (E subunit; E185Q mutation)[21,22,24]. W subunits can cycle through all of the normal nucleotide states (ATP-bound, ADP-bound, empty), whereas the non-hydrolytic E and R

subunits appear to be restricted to a single state. Despite having only two catalytically active subunits and lacking the N-terminal domain of the wild-type enzyme, the RWE/RWE ClpX variant supports ClpP degradation of ssrA-tagged substrates with the same efficiency per active subunit as wild-type ClpX22.

The Ar-Φ motif and substrate recognition

Previous studies showed that the Y153A and V154F mutations in the Ar-Φ motif of homohexameric ClpX abrogated or severely diminished ClpXP degradation activity12. To determine the mechanistic effects of substitutions at these positions in greater detail, we constructed mutations in just the R subunits, just the W subunits, or just the E subunits of RWE/RWE ClpX, purified the mutant enzymes, and determined steady-state K_M and V_{max} values for degradation of the unfolded substrate, titin^{CM}-ssrA. The Y153A, V154F, and V154A mutations increased K_M for degradation of titin^{CM}-ssrA substrates from 3-fold to 60-fold depending on the mutation and subunit type (Table 1; Fig. 1c). For example, V154F increased K_M about 60-fold in W subunits, 10-fold in R subunits, and 3-fold in E subunits. Because the Y153A and V154F mutations were most deleterious in the wild-type subunits of RWE/RWE ClpX, the Ar-Φ pore-loops of hydrolysis-competent subunits seem to play primary roles in binding ssrA-tagged substrates, with support from the Ar-Φ loops of neighboring ATP-bound and/or empty-state subunits.

ATP-hydrolysis control

For each Ar-Φ mutant, we measured the rate of ATP hydrolysis in the presence or absence of saturating substrate and ClpP (Table 1). Some Ar-Φ mutations decreased basal and working ATPase rates, whereas others increased these rates (Table 1; Fig. 2a, 2c). Large increases were observed for mutations in the R and E subunits, despite the fact that these subunits cannot hydrolyze ATP. Hence, ATP hydrolysis is coupled with conformational changes in the Ar-Φ loops of all types of subunits in the hexamer. Mutations with decreased side-chain volumes (Y153A, V154A) tended to increase basal and working ATPase rates, whereas the enzyme with the lowest ATPase rates had a larger mutant side chain (V154F) (Table 1). ClpX conformational changes may be the slow step in the ATP cycle, with tighter packing of residues in the pore resulting in slower conformational transitions and *vice versa*. Similar bidirectional coupling of ATP hydrolysis and conformational changes has been suggested for FtsH and HslU7,14.

Translocation defects

V_{max} for ClpXP degradation of unfolded titin^{CM}-ssrA is determined by the translocation rate18, which is a function of motor speed (the rate of ATP hydrolysis) and the efficiency of each power stroke in transmitting force to the substrate. For one set of enzymes—including the RWE/RWE parent, mutants with the V154F and V154A substitutions in all types of subunits, and the variant with Y153A mutations in the R subunits—we found a roughly constant proportionality between V_{max} for titin^{CM}-ssrA degradation and the working ATP-hydrolysis rate (Fig. 2a). Despite running at considerably different speeds, these class-I mutants and the wild-type parent all hydrolyzed 100–140 ATPs for each molecule of unfolded titin^{CM}-ssrA that was translocated and degraded. Because translocation rates and

ATP-hydrolysis rates were highly correlated, the class-I mutants appear to transmit force to the substrate as well as the wild-type enzyme. Their major defect is in controlling motor speed.

For the Y153A mutation in the W and E subunits of RWE/RWE ClpX, by contrast, substantially more ATP was required to degrade a molecule of titin^{CM}-ssrA. These mutations increased the ATP cost of titin^{CM}-ssrA translocation and degradation almost 3-fold compared to wild type or the class-I mutants (Fig. 2a; Table 1). This need for excess ATP hydrolysis suggests that some power strokes fail to perform useful mechanical work, as might be expected if force is not transmitted to the substrate efficiently. Thus, the aromatic side chains in the Ar-Φ loops of W and E subunits seem to play special roles in substrate translocation, consistent with a role in "gripping" the polypeptide chain during a ClpX power stroke.

Previous studies demonstrated crosslinking of the ssrA tag to the Ar-Φ motif of ClpX under conditions where translocation could not occur[25]. To establish that the first residue of the Ar-Φ motif can directly contact a translocating substrate, as required by the "gripping" model, we replaced the aromatic side chain with cysteine (Y153C) in the W subunit of the WEREER ClpX single-chain hexamer and assayed for disulfide crosslinking to either of two cysteines in an unfolded titin-ssrA substrate. This mutant variant of ClpX, in combination with ClpP, degraded titin^{CM}-ssrA in an ATP-dependent fashion but more slowly than the parental enzyme (not shown). The first substrate cysteine that would be encountered during translocation through the pore is 30 residues from the ssrA tag. Robust crosslinking of the ssrA-tagged substrate to the W^{Y153C}EREER enzyme was observed (Fig. 2b). Several controls established specificity. First, crosslinking did not occur with unfolded titin bearing a mutant ssrA-DD tag that is not recognized by ClpX (Fig. 2b). Second, when the Y153C mutation was placed in the first subunit of EEREER ClpX, which cannot hydrolyze ATP or translocate substrates, efficient crosslinking was not observed (Fig. 2b). Because disulfide bonding requires atomic contact, these results demonstrate that the Ar-Φ pore loop of ClpX can interact directly with a substrate as it is translocated through the working enzyme.

Severe unfolding defects

Unfolding a stable native protein, like titin-ssrA, provides a demanding test of ClpXP function. Simple mechanics ensure that when ClpX pulls on a folded substrate, the substrate resists denaturation and pulls back on the enzyme, amplifying any deleterious consequences of "grip" mutations. The energetic cost of titin-ssrA unfolding is high, even for wild-type ClpXP, because most denaturation attempts fail and many rounds of binding, pulling, and release occur before unfolding is successful[26]. For the parental RWE/RWE enzyme and all V154F variants, unfolding, translocation, and ClpP degradation of a single titin protein required hydrolysis of 1500–2400 ATPs (Table 1; Fig. 2c). By contrast, enzymes with Y153A mutations in R, E, or W subunits hydrolyzed from 10-fold to almost 40-fold more ATP than RWE/RWE during processing of a single titin-ssrA molecule. Because these mutants hydrolyzed far less ATP while degrading unfolded titin^{CM}-ssrA, the excess ATP must be expended during efforts to denature titin. Thus, the aromatic residues in the Ar-Φ

loops of ClpX subunits in all nucleotide states play key roles in increasing the probability of substrate unfolding.

We envision that ATP hydrolysis in a ClpX subunit causes a movement of its Ar-Φ loop, like the power stroke of an oar in rowing. Interactions between the aromatic ring and the substrate would then propel a segment of unfolded polypeptide through the pore (Fig. 3a). Folded substrates cannot enter the narrow ClpX pore, however, and thus power strokes involving the degradation tag would occur against resistance and apply a deforming force to the native protein. If the Y153A mutation in a wild-type subunit weakens interactions between the Ar-Φ loop and substrate, then the resulting loss of grip would affect translocation and unfolding. The unfolding defect would be far more severe than the translocation defect, as we observe, because the strong resisting force during denaturation would break weak interactions between the pore loops and the substrate, allowing Ar-Φ loop movement with little concomitant transmission of force to the substrate. The defects caused by the Y153A mutation would be akin to trying to row a boat with an oar shaft lacking a blade.

Y153A mutations in non-hydrolytic subunits, which cannot power conformational changes, also had large effects on ClpX unfolding (Table 1; Fig. 2c). The Ar-Φ loops in non-hydrolytic subunits might structurally support, guide, or move in concert with the loops of neighboring hydrolyzing subunits. The Ar-Φ loops in non-hydrolytic subunits could also prevent or decrease substrate "slipping" between power strokes. For example, Fig. 3a shows a model in which a hydrolysis-competent subunit cycles through polypeptide binding, translocation, and release steps, while an adjacent subunit binds the polypeptide after the translocation step and before the next power stroke to prevent slipping.

Additional evidence for reduced grip during translocation

As another test for a role of the Ar-Φ loop in "gripping" substrates, we examined degradation of the fusion protein GFP-titin[CM]-ssrA. RWE/RWE ClpXP degrades most of the titin portion of this substrate but stops when it reaches GFP, leaving a 38-residue titin tail that spans the distance from the entrance of the ClpX pore to the active sites in ClpP27. When variants bearing Y153A mutations in R, W, or E subunits were used to attempt ClpXP degradation of GFP-titin[CM]-ssrA, an additional product, corresponding to GFP with a 45-residue tail was produced (Fig. 3b). We note that the 7-residue difference between these two tails need not correspond to any integral number of power strokes or translocation steps because ClpP only cleaves the polypeptide chain of substrates at some amino-acid positions28. These results support a reduced grip on the substrate, leading to slipping once GFP reaches the entrance of the pore or to a reduced ability to pull GFP tightly against ClpX. The ratio of the two truncated products varied for different mutants, consistent with our finding that the Y153A mutation causes more severe defects in some classes of subunits than in others.

Discussion

Taken with previous studies12, our current results show that Y153A mutations in the Ar-Φ pore-loop motif of ClpX have multiple effects. These mutations diminish binding to the ssrA

tag of substrates, change rates of ATP hydrolysis, change rates of translocation and unfolding, and alter the ability of ClpX to pull substrates tightly against the pore. All of these effects may contribute to the fact that ClpX hexamers with the Y153A mutation in each subunit are completely inactive in supporting degradation[12]. Notably, after eliminating mutation-induced effects in substrate binding and ATP hydrolysis, we find that ClpX variants bearing the Y153A substitution are also defective in gripping substrates to drive translocation and unfolding. By contrast, variants bearing the V154F and V154A mutations at an adjacent position in the pore loop are not defective in these activities. Although mutations in the Ar-Φ pore-loop motif of ClpX and other AAA+ ATPases have previously been reported to compromise activity[7,9–17], our current studies provide the first demonstration of a role for this loop in gripping substrates directly.

Asymmetric Ar-Φ loop function in AAA+ unfoldases

In previous studies of the Ar-Φ pore-loop, mutations were present in all six subunits of the hexameric AAA+ enzymes examined[7,9–17]. By using single-chain ClpX variants, however, we have been able to mutate selected pore loops in the hexamer and thus to probe their roles in subunits at different stages of the ATP cycle. We find that mutations in Ar-Φ pore loops of ClpX have distinct effects on ssrA-tag recognition, polypeptide translocation, ATP hydrolysis, and protein unfolding depending on the ability of the host subunits to hydrolyze ATP and/or respond to ATP binding. These results are inconsistent with models in which all six Ar-Φ loops in the central pore of a ClpX hexamer function symmetrically, but suggest instead that individual subunits play specialized roles depending on asymmetric nucleotide states[21,22].

In our experiments, mutations restricted the activities of specific ClpX subunits, but asymmetry must also occur in the wild-type ClpX hexamer, where only some subunits are ATP-bound[21]. Moreover, some crystal structures of AAA+ unfoldases and related homohexameric helicases show asymmetry in structure, nucleotide interactions, and pore-loop conformations of individual subunits[8,9,29,30]. The strong conservation of the Ar-Φ motif and architectural homologies among AAA+ unfolding machines suggest that all of these enzymes will operate by a similar mechanism in which asymmetric conformational movements of specific Ar-Φ loops are transmitted directly to substrates, resulting in protein unfolding and polypeptide translocation through the central pore.

Methods

Protein expression and purification

Linked single-chain variants of ClpX-ΔN (residues 62–424) were constructed by PCR and cloned into pACYCDuet-1 (Novagen), encoding a His$_6$ tag at the N terminus of single-chain trimers and at the C terminus of single-chain hexamers[22]. ClpX variants, ClpP-His$_6$, and titin-I27-ssrA were expressed and purified as described[18,26,31,32]. Titin-ssrA was alkylated for 3 h at 22 °C with a 100-fold excess of iodoacetic acid at pH 8.8 in 6 M GdmCl, resulting in carboxymethylation and unfolding. In titin-DD, the C-terminal AA dipeptide of the ssrA tag is changed to DD. GFP-titin^{CM}-ssrA was obtained by selective unfolding and carboxymethylation of the titin (V15P) domain of the GFP-titin^{V15P}-ssrA fusion protein[31].

Biochemical assays

Degradation of ^{35}S-labeled titin-ssrA and titin^{CM}-ssrA by ClpX- N variants (0.3 mM pseudo-hexamer equivalents) and ClpP$_{14}$ (0.9 mM) was performed at 30 °C in PD buffer (25 mM HEPES (pH 7.6), 100 mM KCl, 20 mM MgCl$_2$, 1 mM EDTA, 10% (v/v) glycerol) with an ATP regeneration system (5 mM ATP, 16 mM creatine phosphate, 6 mg ml^{-1} creatine phosphokinase) and was assayed by the release of acid-soluble peptides18. Degradation of GFP-titin^{CM}-ssrA (20 μM) by ClpX RWE/RWE or variants (1 μM hexamer equivalents) and ClpP$_{14}$ (2 mM) at 30 °C in PD buffer was assayed after 60 min by SDS-PAGE. Partial degradation products were identified by mass spectrometry.

Steady-state ATPase assays were performed using 0.3 mM ClpX-DN variants (hexamer equivalents), in the absence or presence of 0.9 mM ClpP$_{14}$ and saturating amounts of titin-ssrA or titin^{CM}-ssrA at 30 °C in PD buffer with an NADH-coupled regeneration system as described32.

Crosslinking

The two cysteines in titin-ssrA and titin-DD were activated for crosslinking by formation of a mixed disulfide with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). Titin variants (40 μM) were incubated with 1 mM DTNB in 3.5 M GdmCl for 3 h at 22 °C in XL-buffer (25 mM HEPES (pH 7.8), 300 mM KCl, 20 mM MgCl$_2$, 1 mM EDTA, 10% (v/v) glycerol). After separation of free DTNB and GdmCl by buffer exchange, the unfolded, DTNB-activated titin variants (10 mM) were mixed in XL buffer with ClpX W^{Y153C}EREER or E^{Y153C}EREER (1 mM) in the presence of an ATP regeneration system (25 mM ATP, 80 mM creatine phosphate, 30 mg ml^{-1} creatine phosphokinase). Substrate translocation and crosslinking was allowed to proceed for 30 min at 30 °C before stopping the reaction by addition of 150 mM iodoacetic acid in 400 mM Tris-HCl (pH 8.5), 6.2 M urea, 2 mM EDTA. Following non-reducing SDS-PAGE, disulfide-crosslinked titin-ClpX complexes were detected by western blotting using anti-ssrA and anti-DD antibodies.

Acknowledgements

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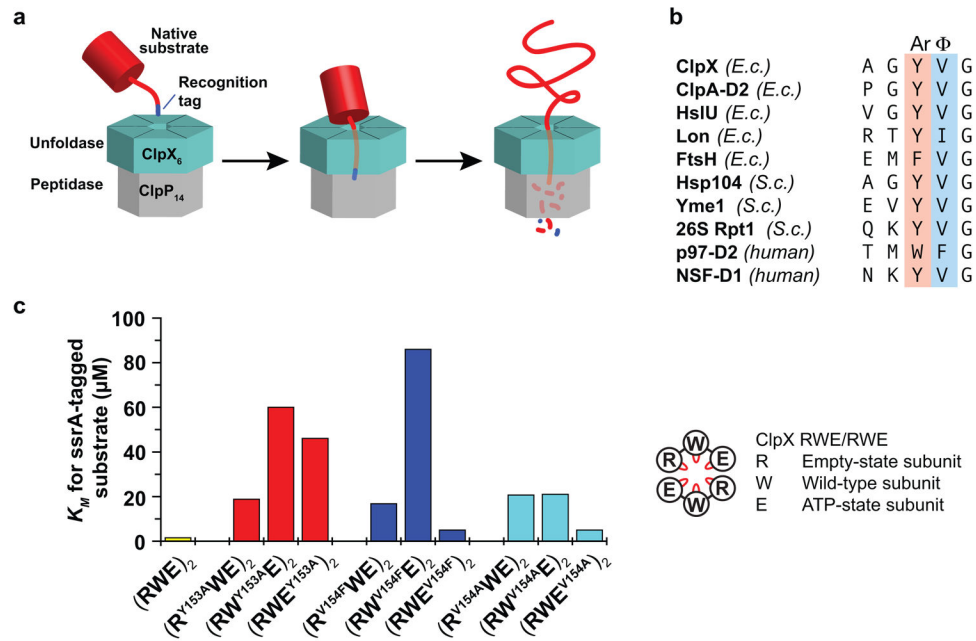
Figure 1. Substrate binding and degradation

(*a*) Native substrates are recognized by the ClpX unfoldase via exposed peptide tags and unfolded as they are translocated through a narrow axial pore and into the ClpP peptidase for degradation. (*b*) Ar-Φ pore-loop motifs in prokaryotic and eukaryotic AAA+ unfoldases. (*c*) Mutations in the Ar-Φ loop of ClpX weaken binding to ssrA-tagged substrates. K_M values for titin^{CM}-ssrA degradation by ClpP in complex with ClpX RWE/RWE or variants with the Y153A, V154F, or V154A mutations in different classes of subunits were determined by Michaelis-Menten analyses of initial degradation rates (Table 1). Errors in K_M were ±10% based on replicate measurements (n=3).
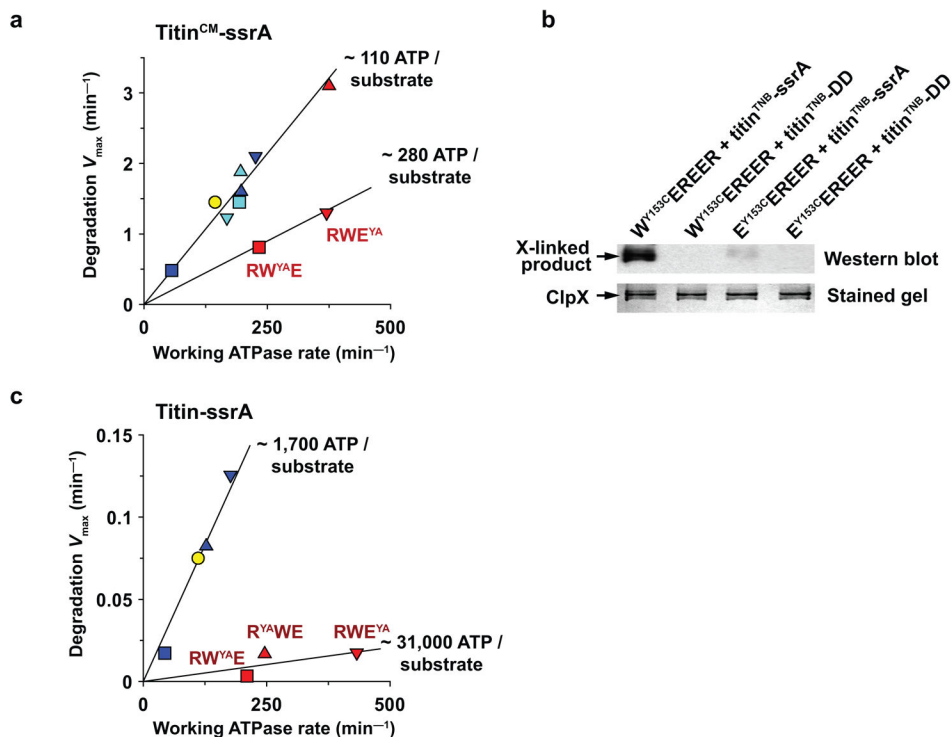
Figure 2. The Ar-Φ loop in the central pore of ClpX provides a "grip" on substrates during unfolding and translocation

(*a*) Thermodynamic efficiencies for titin^{CM}-ssrA translocation by ClpX RWE/RWE with mutations in the Ar-Φ loop. Maximal degradation rates are plotted against the ATP-hydrolysis rate at saturating titin^{CM}-ssrA concentrations for ClpX RWE/RWE (○) and variants with Y153A (red), V154F (blue), or V154A (cyan) mutations in empty-state R subunits (△), hydrolyzing W subunits (□), or ATP-state E subunits (▽). Variants that consume similar amounts of ATP for each substrate degraded cluster close to the lines shown. (*b*) The first residue in the Ar-Φ loop contacts translocating substrates. Titin-ssrA was unfolded by modification of its cysteines with DTNB, incubated with ATP and single-chain ClpX hexamers bearing Cys153 in a W or E subunit, and disulfide-crosslinked products were detected by western blotting after non-reducing SDS-PAGE. DTNB-modified titin with a C-terminal AA→DD mutation in the ssrA tag is not degraded by ClpX and serves as a negative control. (*c*) Thermodynamic efficiencies of native titin-ssrA unfolding and translocation by ClpX RWE/RWE with Ar-Φ-loop mutations. Symbols for ClpX variants are the same as in panel A.
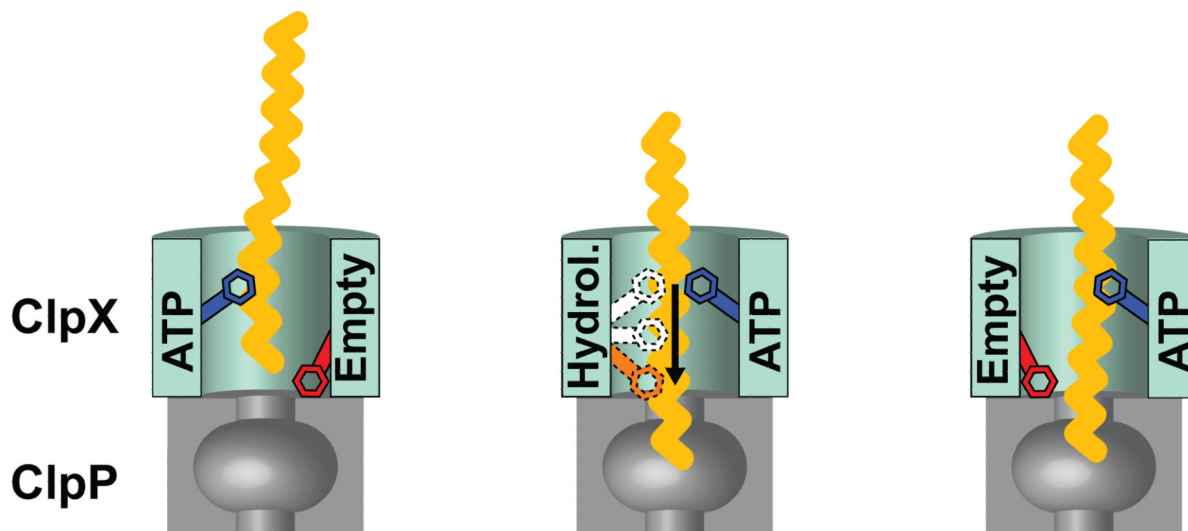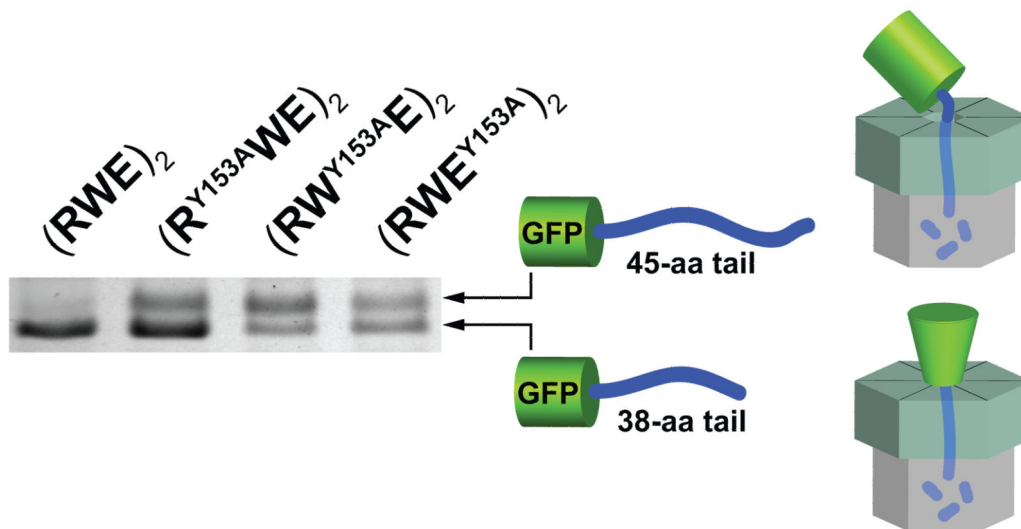
Figure 3. Ar-Φ-loop motions propel substrate through the central pore of ClpX

(*a*) The cartoon depicts two neighboring ClpX subunits cycling through ATP-bound, hydrolyzing, and empty states. ATP-dependent conformational changes in the Ar-Φ loop of one subunit translocate the substrate, and an adjacent subunit binds the polypeptide and prevents slipping before the next power stroke. (*b*) Weakened grip during substrate translocation. Degradation of GFP-titin[CM]-ssrA by ClpXP RWE/RWE stops at GFP, leaving a 38-residue titin tail. Y153A mutations in the R, W, or E subunits of ClpX RWE/RWE result in an additional product with a 45-residue tail. These mutations may reduce the

enzyme's ability to pull GFP tightly against the ClpX pore or allow the substrate to slip after it reaches the pore.

Table 1

Protein-degradation and ATPase activities of Ar-Φ loop mutants of ClpX RWE/RWE.

ClpX variant	basal ATPase (min⁻¹)[a]	titin^{CM}-ssrA				titin-ssrA			
		K_M (μM)[b]	$Vmax$ (min⁻¹)[b]	Working ATPase (min⁻¹)[c]	ATP per substrate[d]	K_M (μM)[b]	V_{max} (min⁻¹)[b]	Working ATPase (min⁻¹)[c]	ATP per substrate[d]
RWE	380	1.5	1.4	140	100	2.1	0.075	110	1,500
R^{Y153A}WE	860	19	3.1	370	120	18	0.017	250	15,000
RW^{Y153A}E	760	60	0.8	230	280	64	0.004	210	55,000
RWE^{Y153A}	880	46	1.3	370	280	40	0.018	430	25,000
R^{V154F}WE	310	17	1.6	200	120	12	0.082	130	1,500
RW^{V154F}E	150	86	0.49	55	110	72	0.018	42	2,400
RWE^{V154F}	410	5	2.1	230	110	4.2	0.13	180	1,400
R^{V154A}WE	420	21	1.9	200	100	21	n.d.	n.d.	n.d.
RW^{V154A}E	390	21	1.5	190	130	20	n.d.	n.d.	n.d.
RWE^{V154A}	890	5	1.2	170	140	7.7	n.d.	n.d.	n.d.

Values are reported to two significant digits.

[a] ATPase activities of ClpX hexamers in the absence of substrate and ClpP. Rates have an error of ±5% based on replicate measurements (n = 3).

[b] K_M and V_{max} values were determined by Michaelis-Menten analyses. Errors are ±10% based on replicate measurements (n = 3).

[c] ATPase activities of ClpX hexamers in the presence of saturating concentrations of protein substrate, ClpP, and ATP. Rates have an error of ±5% based on replicate measurements (n = 3).

[d] ATP molecules hydrolyzed per substrate molecule degraded by ClpXP.