Control of substrate gating and translocation into ClpP by channel residues and ClpX binding

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

| Citation       | Lee, Mary E., Tania A. Baker, and Robert T. Sauer. “Control of Substrate Gating and Translocation into ClpP by Channel Residues and ClpX Binding.” Journal of Molecular Biology 399.5 (2010): 707–718. |
|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| As Published   | http://dx.doi.org/10.1016/j.jmb.2010.04.027                                                                                                                                                      |
| Publisher      | Elsevier                                                                                                                                                                                        |
| Version        | Author’s final manuscript                                                                                                                                                                       |
| Citable link   | http://hdl.handle.net/1721.1/72470                                                                                                                                                            |
| Terms of Use   | Creative Commons Attribution-Noncommercial-Share Alike 3.0                                                                                                                                 |
| Detailed Terms | http://creativecommons.org/licenses/by-nc-sa/3.0/                                                                                                                                            |
Control of substrate gating and translocation into ClpP by channel residues and ClpX binding

Mary E. Lee¹, Tania A. Baker¹,², and Robert T. Sauer¹,*
¹Department of Biology Massachusetts Institute of Technology Cambridge, MA 02139 USA
²Howard Hughes Medical Institute Massachusetts Institute of Technology Cambridge, MA 02139 USA

Abstract

ClpP is a self-compartmentalized protease, which has very limited degradation activity unless it associates with ClpX or ClpA to form the AAA+ ClpXP or ClpAP proteases. Here, we show that ClpX binding stimulates ClpP cleavage of peptides larger than a few amino acids and enhances ClpP active-site modification. Stimulation requires ATP binding but not hydrolysis by ClpX. The magnitude of this enhancement correlates with increasing molecular weight of the molecule entering ClpP. Amino-acid substitutions in the channel loop or helix A of ClpP enhance entry of larger substrates into the free enzyme, eliminate ClpX binding in some cases, and are not further stimulated by ClpX binding in other instances. These results support a model in which the channel residues of free ClpP exclude efficient entry of all but the smallest peptides into the degradation chamber, with ClpX binding serving to relieve these inhibitory interactions. Specific ClpP channel variants also prevent ClpXP translocation of certain amino-acid sequences, suggesting that the wild-type channel plays an important role in facilitating broad translocation specificity. In combination with previous studies, our results indicate that collaboration between ClpP and its partner ATPases opens a gate that functions to exclude larger substrates from isolated ClpP.

INTRODUCTION

Intracellular protein degradation is an important facet of proteome maintenance, and misregulation of proteolysis can lead to severe cellular defects.¹–⁶ One mechanism that helps ensure that only the proper proteins are degraded is to assemble cytoplasmic proteases into self-compartmentalized structures in which the active sites for peptide-bond cleavage are sequestered within the lumen of a barrel-shaped chamber, which is only accessible through narrow axial channels or pores.⁷–⁸ Proteins that are substrates of such enzymes are typically recognized by an AAA+ ATPase that is associated with the compartmental protease, unfolded if necessary, and then translocated into the proteolytic chamber.⁹

ClpP is a self-compartmentalized protease, formed by the stacking of two heptameric rings.¹⁰–¹¹ It associates with AAA+ hexamers of either ClpX or ClpA to form the ATP-dependent proteases, ClpXP or ClpAP.⁷–⁸ By itself, ClpP does not degrade native or denatured proteins.¹²–¹³ Thus, mechanisms must exist to limit the destructive potential of this enzyme. The
importance of proper regulation of ClpP activity is highlighted by the action of acyldepsipeptide antibiotics, which kill bacteria by binding to ClpP, preventing association with ClpX or ClpA, and endowing ClpP with the ability to degrade unfolded polypeptides, including nascent chains.\(^{14-15}\) Two distinct mechanisms can limit degradation by other self-compartmentalized proteases in the absence of a AAA+ partner. One mechanism involves active-site remodeling. In the HslUV system, for example, the active-site residues of HslV assume an inactive conformation and fail to cleave even small peptides or to react with vinyl-sulfone inhibitors, when its AAA+ HslU partner is absent.\(^{16-19}\) In this instance, binding of an HslU ring to an HslV ring drives remodeling of the active site into a functional conformation.\(^{19-20}\) Another mechanism involves gating of substrate access to the proteolytic chamber. For example, structures reveal functional active sites in the chamber of the isolated 20S compartmental protease of the eukaryotic proteasome but also show that substrate access to these sites is severely limited by residues which sterically block the entrance pore.\(^{21-23}\) This blockade is relieved by structural rearrangements that accompany binding of the 20S core to the 19S regulatory complex or to non-ATPase regulators such as PA26.\(^{24-25}\)

We are interested in the mechanism(s) that repress the proteolytic activity of the isolated ClpP enzyme and allow activation by ClpX. \(Escherichia \ coli\) ClpP is initially expressed as a proenzyme, which is autoproteolytically processed to remove an N-terminal propeptide.\(^{10}\) Crystallographic studies of the mature \(E. \ coli\) ClpP tetradecamer reveal canonical Ser-His-Asp catalytic triads and a properly formed oxyanion hole, which appear to be functional as free ClpP can degrade small peptides and reacts with diisopropylfluorophosphate, a covalent active-site inhibitor.\(^{11-13}\) However, in structures of ClpP from other species, the catalytic triads appear to be functional in some cases and malformed in other instances, raising the possibility that active and inactive conformations of ClpP equilibrate in solution.\(^{7,26-31}\) The axial channel of free ClpP is formed by N-terminal stem-loop structures, with the stems forming the rim of the pore and the loops forming the channel.\(^{11,26-31}\) This channel is too narrow to admit native proteins and peptides of any substantial size are also degraded very slowly.\(^{32-33}\) However, deletion of segments of the pore and channel allow degradation of unfolded proteins, which are not degraded by wild-type ClpP alone.\(^{34}\) Moreover, ClpP degradation of large peptides can be stimulated substantially by ClpA binding.\(^{13}\) These results are consistent with regulation of ClpP proteolytic activity by a simple gating mechanism. However, recent studies suggest that regulation of the active-site conformation of ClpP by ClpX or ClpA binding may also be required under some circumstances to allow hydrolysis of the acyl intermediate in peptide-bond cleavage.\(^{35}\) Electron microscopy (EM) shows that hexameric rings of ClpX or ClpA stack coaxially with the heptameric rings of ClpP, aligning the central translocation channel of the AAA+ ATPase with the ClpP pore.\(^{32-36}\) There are no crystal structures of ClpXP or ClpAP, however, and the resolution of the EM structures are insufficient to observe atomic details. It is known that formation of these active proteolytic complexes requires ATP or ATPgS, affects the rate of ATP hydrolysis, and requires conserved IGF/IGL motifs located in loops on the AAA+ ring that appear to dock into hydrophobic clefts on the periphery of the ClpP ring.\(^{32-34,37-41}\) Amino acids that form the axial pore and channel of ClpP also appear to play roles in recognition of the AAA+ rings of ClpX and ClpA.\(^{26-28,34,41}\)

In this paper, we test predictions of the pore-gating model for ClpP and investigate the role of ClpX in controlling gating. We find that ClpX binding stimulates ClpP cleavage of peptide substrates larger than a few amino acids in a reaction that does not require ATP hydrolysis. Moreover, this stimulatory effect increases as a function of peptide molecular weight, as expected if ClpX binding increases the rate at which peptides diffuse into ClpP. ClpX binding also stimulates active-site modification of ClpP by fluorophosphates, but only to a level expected from faster diffusion of the inhibitor into the ClpP chamber. The ability of wild-type ClpP to exclude large peptides depends on interactions mediated by the channel region of the pore and by conserved residues in \(\alpha\)-helix A. Mutations in the ClpP channel and helix A also
weaken ClpX binding and affect degradation efficiency. Importantly, we find that ClpP channel mutations can prevent ClpXP translocation of certain amino-acid sequences, suggesting that the wild-type ClpP channel has evolved to allow broad translocation specificity.

RESULTS

Size-dependent ClpX stimulation of ClpP active-site reactivity and peptide cleavage

In initial experiments, we used a rhodamine-labeled fluorophosphate inhibitor (Rh-FP; Mr 845 Da) to probe the reactivity of the active-site serines of E. coli ClpP, both alone and in complex with E. coli ClpX. For these experiments, the fluorescent inhibitor was incubated with ClpP, with ClpP plus ClpX•ATPγS, or with ClpP plus ClpX•ADP for different times before quenching the reaction. Samples were then analyzed by SDS-PAGE and fluorimetry. In the ClpX•ATPγS experiment, the rate of covalent active-site modification by Rh-FP was ~3-fold faster than for ClpP alone (Fig. 1A). The rate of modification with ClpX•ADP, which does not bind ClpP, was the same as that measured for ClpP alone.

Prior studies demonstrated that ClpA did not stimulate ClpP cleavage of very small peptides but did stimulate cleavage of longer peptides. To test this possibility for ClpX, we assayed the dependence of ClpP cleavage of a dipeptide (succinyl-LY-AMC; Mr 552 Da), a decapetide (Mr 1219 Da), and an icoseptide (Mr 2404 Da). The 10- and 20-residue substrates were flanked by a fluorophore (2-aminobenzoic acid) and quencher (3-nitrotyrosine) to allow cleavage to be detected by increased fluorescence. The rates of dipeptide cleavage by ClpP alone or in the presence of ClpX•ATPγS were within error (Fig. 1B). By contrast, ClpXP•ATPγS cleaved the decapetide ~9-fold faster and the 20-residue substrate ~40-fold faster than ClpP by itself (Fig. 1B).

ClpX can use ATPγS hydrolysis, which occurs ~10-fold more slowly than ATP hydrolysis, to power protein unfolding, translocation, and degradation. To test if nucleotide hydrolysis by ClpX was required for enhanced ClpP cleavage of the decapetide substrate, we also determined rates in the presence of ClpX•ATP and ClpX[E285Q]•ATPγS. The ClpX[E285Q] mutant harbors a substitution for a highly conserved glutamate in the Walker B motif and is defective in ATP hydrolysis although it still binds ClpP in an ATP-dependent fashion. In both cases, decapetide cleavage occurred at similar rates to those observed with ClpX•ATPγS (Fig. 1C). No stimulation of cleavage was observed with ClpX•ADP (Fig. 1C). We conclude that nucleotide hydrolysis is not essential for ClpX stimulation of peptide degradation by ClpP.

Fig. 1D shows that activity stimulation by ClpX increases as a function of the molecular weight of the compound that needs to enter ClpP. These findings, in conjunction with previous results, support a model in which ClpX binding to ClpP facilitates faster diffusion of Rh-FP, the decapetide, and the icoseptide into the proteolytic chamber of ClpP. Because the dipeptide substrate seems to enter the chambers of ClpP and ClpXP at comparable rates, it appears that the pore in isolated ClpP only restricts entry of compounds with a higher molecular weight or larger radius of gyration.

Substitution mutations in the ClpP channel activate peptide cleavage

Residues 8–15 of wild-type E. coli ClpP form the channel loop of the pore (Fig. 2A), and include four highly conserved charged residues (EQTSRGER). N-terminally truncated ClpP variants, lacking channel-loop residues, show faster degradation of large peptides, raising the possibility that substitution mutations might also alter ClpP’s ability to discriminate against longer peptides. Indeed, when we replaced residues 8–15 with eight glycines, this mutant (GGGGGGGGG) cleaved the decapetide at a rate ~8-fold faster than that observed with wild-type ClpP (Fig. 2B) but cleaved the dipeptide at a comparable rate (Fig. 2C). Similar results
were observed for mutants in which the charged residues in the channel were reversed (RQTS\textit{EGRE})\textsuperscript{15}, replaced by glycines (GQTSGGGG\textsuperscript{15}), or replaced by alanines (AQTSAGAA\textsuperscript{15}) (Fig. 2B & 2C). Mutations in the N-terminal loop can alter ClpP processing.\textsuperscript{28} However, all of these channel-substitution mutants were properly processed by the criteria that they had the same mobility as wild-type ClpP during SDS-PAGE. For the GGGGGGGGG\textsuperscript{15} mutant, MALDI-TOF mass spectrometry also gave the expected molecular weight. In combination, these results suggest that proper regulation of access to the proteolytic chamber of ClpP requires interactions mediated by charged residues in the wild-type channel.

**Channel residues influence ClpX binding and ClpXP degradation**

To assess the effects of channel mutations on ClpX interactions, we titrated increasing quantities of the mutant variants or wild-type ClpP against a fixed concentration of ClpX and assayed degradation of GFP-ssrA. The ssrA tag targets substrates to the axial pore of ClpX, and unfolding and subsequent degradation of GFP-ssrA require ATP hydrolysis.\textsuperscript{41,46–47} As assayed by loss of native fluorescence, substantial GFP-ssrA degradation was observed in the experiments using wild-type ClpP or AQTSAGAA\textsuperscript{15} ClpP but much slower degradation was detected using the RQTS\textit{EGRE}, GQTSGGGG\textsuperscript{15}, and GGGGGGGG\textsuperscript{15} ClpP variants (Fig. 2D). Moreover, higher concentrations of the AQTSAGAA\textsuperscript{15} mutant than of wild-type ClpP were required for half-maximal stimulation of proteolysis, and the GFP-ssrA degradation rate at saturation was approximately two-fold slower for the AQTSAGAA\textsuperscript{15} mutant than for wild-type ClpP (Fig. 2D). We conclude that residues in the ClpP channel play important roles in binding ClpX, in determining the maximal rate of degradation of protein substrates, and in regulating ClpP gating.

Decapeptide cleavage by ClpP was stimulated roughly 10-fold by ClpX binding or by the AQTSAGAA\textsuperscript{15} channel mutations. If these channel mutations and ClpX binding affected ClpP activity independently, then their combined effects would be expected to be roughly additive. However, when we combined a saturating concentration of ClpX with AQTSAGAA\textsuperscript{15} ClpP, almost no additional increase in decapeptide cleavage activity was observed (Fig. 2E). This result suggests that these channel mutations and ClpX binding affect ClpP gating in generally similar fashions.

**ClpP-channel residues facilitate translocation of specific substrate sequences**

We also tested degradation of a 33-residue ssrA-tagged peptide with a YGYGYGYGYG guest region (see ref. 48) by the ClpP channel-loop mutants in combination with ClpX and ATP. To limit degradation of this substrate (called [YG]\textsubscript{5}-ssrA) via diffusion into the ClpP proteolytic chamber, we added the SspB protein, which binds part of the ssrA tag and restricts the bound peptide from entering ClpP unless ClpX is present and can interact functionally with ClpP.\textsuperscript{48–51} Degradation of this peptide by ClpP or the AQTSAGAA\textsuperscript{15} mutant increased as a function of ClpX concentration and saturated, but half-maximal degradation by AQTSAGAA\textsuperscript{15} ClpP required an approximate ~10-fold higher concentration of ClpX and the degradation rate was ~six-fold slower when ClpX was saturating (Fig. 3A). The highest ClpX concentrations tested did not stimulate degradation of the [YG]\textsubscript{5}-ssrA peptide by RQTSEG\textsubscript{15} or GQTSGGGG\textsuperscript{15} ClpP (Fig. 3A).

Intriguingly, AQTSAGAA\textsuperscript{15} ClpXP degraded native GFP-ssrA at ~50% of the wild-type rate but only degraded the unfolded [YG]\textsubscript{5}-ssrA peptide at ~20% of the corresponding wild-type velocity (Fig. 2D & 3A). We also tested degradation of additional ssrA-tagged peptides with different guest sequences for degradation by AQTSAGAA\textsuperscript{15} ClpXP as well as wild-type ClpXP (Fig. 3B). Surprisingly, AQTSAGAA\textsuperscript{15} ClpXP showed almost no degradation of peptides with 10 glutamic acids, 10 lysines, or four glycines in the guest region. Somewhat higher rates were observed for peptides with guest regions containing 10 glutamines, 10
arginines, or six alanines (Fig. 3B). AQTSAGAA ClpXP degraded peptides with guest regions containing proline or repeats of tyrosine-glycine or phenylalanine-glycine at the highest rates. In all cases, wild-type ClpXP degraded the same peptides substantially faster (Fig. 3B). Thus, mutating the charged residues in the wild-type ClpP channel to alanines seems to restrict translocation of charged, polar, and small amino acids more than it affects translocation of more hydrophobic side chains. Therefore, the chemical identities of the amino acids that form the wild-type channel appear to facilitate broad translocation specificity.

Helix-A residues play roles in substrate gating

In a screen for dominant-negative ClpP mutations that prevent or reduce wild-type ClpXP activity, we isolated I19T and S21Y mutations in helix A (in preparation). This helix, which consists of residues 19–25, is immediately proximal to the N-terminal channel loops and pore in the ClpP structure (Fig. 4A), and Ile<sup>19</sup> and Leu<sup>24</sup> in helix A form a hydrophobic cluster with Phe<sup>49</sup> and pore-stem residues Pro<sup>4</sup> and Val<sup>6</sup> (Fig. 4B). To investigate the role of helix A in ClpP activity, we constructed alanine-substitution mutations at each helix-A residue, purified the mutant enzymes, and assayed peptidase activity. All of these mutants showed essentially wild-type levels of dipeptide cleavage (not shown), but their rates of decapeptide cleavage differed substantially (Fig. 4C). Relative to wild-type ClpP, for example, the R22A and K25A mutations caused small increases (~1.5 fold) in decapetide cleavage, the Y20A, S21A, and L23A mutations caused modest increases (~5 fold), and the I19A and L24A mutations caused large increases (~20 fold). We also constructed and purified mutants with more conservative leucine and valine substitutions for Ile<sup>19</sup>. Decapetide cleavage was increased ~6 fold by the I19L mutation and ~16 fold by the I19V mutation (Fig. 4D). Thus, even subtle changes in the stereochemistry of the side chain of residue 19 alter ClpP gating.

We used trypsin to probe effects of the helix-A alanine-substitution mutations on the conformation of the ClpP channel loops, which contains potential sites of tryptic cleavage after Arg<sup>12</sup> and Arg<sup>15</sup>. Incubation of a wild-type ClpP variant bearing a C-terminal His<sub>6</sub>-tag with trypsin resulted in formation of a stably truncated fragment within 30 min (Fig. 5A). Control experiments showed that cleavage of the C-terminal His<sub>6</sub>-tag was responsible for this truncation. The Y20A, S21A, R22A, L23A, and K25A mutants behaved like the wild-type control after incubation with trypsin (Fig. 5A). By contrast, the I19A and L24A mutations modestly enhanced trypsin susceptibility, as shown by lower molecular-weight products following SDS-PAGE (Fig. 5A). Edman sequencing of these smaller fragments revealed that the I19A mutant was cleaved after Arg<sup>12</sup>, whereas the L24A mutant was cleaved after Arg<sup>15</sup> (data not shown). Thus, the I19A and L24A mutations, which resulted in the largest increases in decapetide cleavage, also increase the susceptibility of the N-terminal ClpP channel loop to tryptic cleavage. These results support a model in which packing interactions mediated by the wild-type side chains of Ile<sup>19</sup> and Leu<sup>24</sup> stabilize a stem-loop conformation of the N-terminal channel that restricts passage of all but the smallest peptides into free ClpP.

If helix-A mutations increase decapetide cleavage solely by influencing the conformation of the ClpP channel and pore, then the effects of a double mutation involving helix A and the channel should be non additive. Indeed, when we constructed and purified an L24A/GGGGGGGG<sup>15</sup> variant of ClpP, this double mutant was no more active in decapetide cleavage than the parental L24A or GGGGGGGG<sup>15</sup> mutants (Fig. 5B). These results strongly suggest that mutations in both helix A and in the ClpP channel disrupt interactions required to restrict diffusion of the decapetide substrate into the ClpP proteolytic chamber.

Deletion of the N-terminal 10, 14, or 17 residues of mature ClpP allows these mutants to degrade α-casein, a natively unfolded protein. To test if a helix-A mutation also allows ClpP to degrade unfolded proteins, we assayed degradation of a carboxymethylated and <sup>35</sup>S-labeled variant of the I27 domain of human titin. As shown in Fig. 5C, this 113-residue unfolded
substrate was degraded efficiently by I19A ClpP and showed little or no degradation by wild-type ClpP. The I19A ClpP mutant did not degrade the native titin I27 domain (not shown). These results demonstrate that a single point mutation in ClpP is sufficient to allow degradation of unfolded proteins and highlight the important role of I19 in controlling access to the ClpP chamber.

**Helix-A mutations disrupt ClpX binding**

Deletion and substitution mutations affecting residues that form the ClpP pore and channel have been shown to weaken or prevent ClpX binding. To test if the alanine-substitution mutations in helix A have similar effects, we combined increasing quantities of these mutants with a fixed concentration of ClpX and assayed for ATP-dependent degradation of GFP-ssrA. The I19A, Y20A, S21A, R22A, and L24A mutants showed very low substrate cleavage at the highest ClpP concentrations tested, suggesting substantial defects in ClpX binding, whereas the L23A and K25A mutants showed only modest decreases in apparent ClpX affinity (Fig. 6A). Similar results were obtained when interactions with the ClpP mutants were assayed by changes in ClpX ATP hydrolysis; the L23A and K25A mutants showed modest reductions in affinity, whereas no substantial interactions with the remaining helix-A mutant were detected (not shown).

How do the effects of helix-A mutations on ClpX interactions correlate with decapeptide cleavage or trypsin susceptibility? For the I19A and L24A mutations, all three assays showed large changes compared to the wild-type controls. For the S21A and R22A mutations, by contrast, large effects on apparent ClpX binding were coupled with relatively small effects on decapeptide cleavage and no changes in trypsin susceptibility. Similarly, the K25A mutant bound ClpX far better than the R22A mutant, but both mutations resulted in similar rates of decapeptide cleavage (Fig. 4C). Thus, these results suggest that helix-A mutations, such as S21A and R22A, influence ClpX binding directly rather than exclusively through indirect effects on ClpP-channel residues.

We also tested the ability of the S21A and R22A mutants to degrade GFP-ssrA in collaboration with ClpA (Fig. 6B). In both cases, these mutants bound ClpA far more strongly than they bound ClpX. We could not determine accurate binding constants, but each curve in Fig. 6B was calculated assuming an affinity of 10 nM, suggesting that these mutants bind ClpA roughly as well as wild-type ClpP. At saturation, R22A ClpAP degraded GFP-ssrA at nearly the same rate as wild-type ClpAP, whereas S21A ClpAP degraded this substrate at roughly two-thirds of the wild-type rate (Fig. 6B). These results demonstrate that the S21A and R22A ClpP mutants can collaborate with a partner ATPase in unfolding, translocation, and degradation of a native protein substrate. We conclude that the Arg22 and Ser21 side chains of ClpP play more important roles in binding ClpX than in binding ClpA.

**DISCUSSION**

Previous studies demonstrated that ClpP alone has a very limited ability to degrade peptide substrates larger than a few amino acids, but this activity could be stimulated substantially by ClpA. The work reported here shows that ClpX binding to ClpP also enhances its rate of polypeptide degradation. For both ClpX and ClpA, the magnitude of the rate enhancement increases with the molecular weight of the peptide substrate and requires ATP binding by the AAA+ enzyme but not hydrolysis. These results in combination with crystal structures of ClpP and studies of ClpP mutants suggest that simple ATP-dependent binding of either ClpX or ClpA to ClpP induces conformational rearrangements that allow unfolded peptide and polypeptide substrates to diffuse through the axial channel and into the proteolytic chamber of ClpP. Although ClpX and ClpA are both AAA+ ATPases, they differ markedly in size,
sequence, substrate specificity, and some aspects of their interactions with ClpP.\textsuperscript{38,49,53--55} Thus, their common ability to activate ClpP peptide cleavage was not a foregone conclusion.

The majority of ClpP molecules in \textit{E. coli} appear to be bound to ClpX or ClpA, but measurements of intracellular abundance and affinity calculations suggest that some ClpP is present as the free enzyme.\textsuperscript{56} The inability of this uncomplexed ClpP to degrade unstructured polypeptides is likely to be important in avoiding rogue degradation of such sequences in the cell. For example, unstructured regions can be essential for protein function, native and denatured proteins are often in dynamic equilibrium, and nascent polypeptides might be degraded immediately after synthesis. Indeed, acyldepsipeptide antibiotics kill \textit{E. coli} and other strains of bacteria by activating ClpP polypeptide degradation and preventing its binding to ClpX/ClpA.\textsuperscript{14,15} Our results indicate that restriction of the polypeptide cleavage activity of free ClpP depends both on the identity of residues in the wild-type channel and in the neighboring helix A.

ClpX- or ClpA-dependent remodeling of the ClpP channel is probably required to allow efficient ATP-fueled degradation by these AAA+ proteases. Degradation of unfolded polypeptides by ClpXP or ClpAP, which is independent of ATP hydrolysis, is a useful biochemical assay but is likely to be biologically irrelevant. This conclusion follows from the facts that only ATP-bound forms of ClpX or ClpA bind ClpP and that ATP hydrolysis by these complexes is both constitutive and stimulated by substrates.\textsuperscript{12,40,45,52} In fact, wild-type ClpXP hydrolyzes roughly 100 ATPs during degradation of a single molecule of the unfolded titin I27 domain.\textsuperscript{52}

Deletion of N-terminal sequences that form the ClpP channel has been shown to activate polypeptide cleavage, and crystal structures of mutants lacking 14 or 17 N-terminal residues reveal altered positions for some of the remaining pore/channel residues.\textsuperscript{34--35} Our results show that amino-acid substitutions in helix A (e.g., I19A) and in the channel (e.g. AQTSAGAA\textsuperscript{15}) have similar effects to those caused by large N-terminal deletions in terms of allowing ClpP degradation of polypeptide substrates. These results suggest that relatively small perturbations in the structure of ClpP are sufficient to permit more efficient polypeptide degradation. Systems of this type, in which many different mutations lead to similar gain-of-function phenotypes, generally occur via an increase in the population of an active allosteric conformation because a competing inactive conformation is destabilized. For ClpP, it seems likely that “restrictive” and “permissive” conformations of the channel are in dynamic equilibrium in the wild-type enzyme, with the permissive conformation being present in just a small fraction of enzymes. By this model, any mutation that destabilized the restrictive conformation would increase the population of enzymes with permissive channels.

In the simplest allosteric model, activating ClpP mutations and the binding of ClpX or ClpA might stabilize the same permissive ClpP conformation. In this case, however, activating mutations should enhance binding of the AAA+ ATPases to ClpP, because less binding energy would be required to drive the conformational change. This result is not observed. Indeed, our work and previous studies\textsuperscript{34} show that activating channel deletion and substitution mutations reduce ClpP affinity for ClpX and ClpA. Although it is formally possible that all of the mutations that activate polypeptide cleavage by ClpP also involve side chains that directly contact the AAA+ ATPases in the ClpXP or ClpAP complexes, this explanation seems unlikely. The observed results could also be explained if there were multiple “permissive” conformations of the ClpP channel and ClpX or ClpA binding stabilized different conformations than the activating mutations. High-resolution structures of ClpP in complex with ClpX or ClpA will be needed to resolve this issue.
There is abundant evidence that ClpX and ClpA interact with ClpP channel residues. For example, both double-mutant cycle analysis and crosslinking support the existence of contacts between the axial pore-2 loops of ClpX and channel residues of ClpP.\(^{41}\) We find that certain helix-A mutations (S21A; R22A) also effectively eliminate binding to ClpX. Although remodeling of channel residues could indirectly cause some of these helix-A effects, our results are most consistent with direct effects on ClpX binding. For example, the R22A mutation causes a far larger defect in ClpX binding than in ClpA binding, activates ClpP polypeptide cleavage only modestly, and does not alter the sensitivity of ClpP channel residues to tryptic cleavage. In the ClpP structure, helix A connects the pore and channel residues to residues that form the hydrophobic clefts, which serve as docking sites for the IGF/IGL motifs of ClpX and ClpA. It remains to be determined if these docking interactions transmit a signal via helix A that remodels the channel residues of ClpP or if other binding interactions are responsible for this activity. In this regard, interactions between the pore-2 loops of ClpX and ClpP are not required for activation of polypeptide cleavage.\(^{41}\)

We also found that substitution mutations in the ClpP channel appear to prevent translocation of certain substrate sequences during ATP-dependent degradation by ClpXP. Specifically, replacing Glu\(^8\), Arg\(^{12}\), Glu\(^{14}\), and Arg\(^{15}\) in the wild-type ClpP channel with alanines resulted in a mutant, which in combination with ClpX, could degrade ssrA-tagged peptides with guest regions consisting of YGYGYGYGYG or FGFGFGFGFG sequences but could not degrade otherwise identical peptides containing runs of glutamic acids, lysines, or glycine only. These findings indicate that the wild-type ClpP channel facilitates translocation of diverse substrate sequences, including highly charged amino acids, during normal ClpXP function and thus plays an important role in ensuring that any substrate that can be unfolded and translocated by ClpX can also be degraded by ClpP.

**MATERIALS AND METHODS**

**Proteins and peptides**

The *E. coli* ClpP variants used in these studies had C-terminal His\(_6\) tags, were generated by inverse PCR mutagenesis, and were expressed from multi-copy, IPTG-inducible pQE70 vectors, and purified as described.\(^{46}\) Mutant names refer to the altered amino-acid position(s) in the mature form of *E. coli* ClpP. Wild-type *E. coli* ClpX, and covalently linked wild-type ClpX trimers lacking the N-domain were expressed and purified as described.\(^{45,52,57}\) ClpX\(^{E185Q}\) was a gift from G. Hersch (MIT).

Succinyl-LY-AMC was purchased from Sigma. The Abz-KASPVSGLGY\(^{NO2}\) decapetide (where Abz is the fluorophore 2-aminobenzoic acid and Y\(^{NO2}\) is the quencher 3-nitrotyrosine) was a gift from B. Cezairliyan (MIT). The Abz-ASSHATRQLSGLKIHSNLY\(^{NO2}\) icosapeptide was a gift from Eyal Gur (MIT). SsrA-tagged peptides were gifts from Igor Levchenko and Sarah Barkow (MIT) and consisted of an N-terminal cleavage module (Abz-FAPHMALVPY\(^{NO2}\)), a guest region ([YG]\(_5\), [FG]\(_5\), [E]\(_{10}\), [R]\(_{10}\), [K]\(_{10}\), [Q]\(_{10}\), [A]\(_6\), [P]\(_5\) or [G]\(_4\)) and a C-terminal sequence (KKANDENYALAA) containing the ssrA tag.\(^{48}\) GFP-ssrA was purified as described.\(^{58}\) *E. coli* SspB was a gift from S. Barkow (MIT).

**Assays**

Degradation assays were performed at 30 °C in PD buffer, which consists of 25 mM HEPES (pH 7.6), 100 mM KCl, 20 mM MgCl\(_2\), 1 mM EDTA, and 10% glycerol, and were generally monitored by changes in fluorescence using a QM-2000-4SE spectrofluorimeter (Photon Technology International). When ClpX was present in assays, it was preincubated with 1–5 mM nucleotide (ATP, ATP\(_{γ}\)S, or ADP) for at least 1 min prior to addition of ClpP and substrate. Unless noted, peptide-cleavage assays contained 0.3 μM ClpP\(_{14}\) with or without 0.5 μM

*J Mol Biol.* Author manuscript; available in PMC 2011 June 25.
ClpX<sub>6</sub> and were monitored by changes in fluorescence (dipeptide, excitation 345 nm, emission 440 nm; decapeptide and icosapeptide, excitation 320 nm, emission 420 nM). All peptide-cleavage assays used substrate concentrations substantially below \( K_\text{M} \) for degradation by ClpP<sub>14</sub>, as shown by linear changes in rate versus substrate concentration plots. Degradation of ssrA-tagged peptides (excitation 320 nm; emission 420 nm) was assayed using 8 \( \mu \text{M} \) substrate, 8 \( \mu \text{M} \) E. coli SspB, 4 \( \mu \text{M} \) ClpP<sub>14</sub>, 0.15 \( \mu \text{M} \) ClpX<sub>6</sub>, and an ATP regeneration mix. Control experiments lacking ClpX were performed to test background peptide degradation by free ClpP. Under these conditions, the substrate concentration is well above \( K_\text{M} \). For GFP-ssrA degradation assays (excitation 467 nm; emission 511 nm), each reaction contained 5 \( \mu \text{M} \) substrate (<1–2 \( \mu \text{M} \)), a covalently linked ClpX-\( \Delta \text{N} \) trimer (0.1 \( \mu \text{M} \) pseudo-hexamer equivalents) or ClpA<sub>6</sub> (50 nM), an ATP regeneration mix, and increasing concentrations of ClpP or ClpP mutants. For ClpX-independent degradation of the unfolded protein, carboxymethylated \(^{35}\text{S}\)-titin-I27-ssrA, 5 \( \mu \text{M} \) substrate was incubated with 1 \( \mu \text{M} \) ClpP<sub>14</sub> or I19A ClpP<sub>14</sub> and degradation was monitored by the release of acid-soluble peptides.\(^{52}\) Active-site modification of ClpP<sub>14</sub> (0.5 \( \mu \text{M} \)) with rhodamine-FP (200 \( \mu \text{M} \); a gift of B. Cravatt, Scripps) was performed in PD buffer at 0° C with or without ClpX<sub>6</sub> (1 \( \mu \text{M} \)), ADP (5 mM), or ATP\( \gamma \text{S} \) (5 mM). Reactions were quenched in 2% SDS, separated by SDS-PAGE on 12% gels, and fluorescently modified ClpP was quantified using a Typhoon fluorimeter and ImageQuant software. Tryptic digests were performed in PD buffer at 30 °C using wild-type or mutant ClpP<sub>14</sub> (0.7 \( \mu \text{M} \)) and porcine pancreas trypsin (0.5 \( \mu \text{M} \); Sigma). At different times, reactions were quenched by boiling in 2% SDS and 1 mM phenylmethanesulphonyl fluoride, and aliquots were subjected to 12% SDS-PAGE and then stained with Coomassie Blue.

Acknowledgments

We thank S. Barkow, B. Cezairliyan, B. Cravatt, E. Gur, G. Hersch, D. Jerulzami, A. Keating, I. Levchenko, and P. Sharp for helpful discussions and/or reagents. Supported in part by NIH grant AI-15706. TAB is an employee of the Howard Hughes Medical Institute.

REFERENCES

1. Gottesman S. Proteases and their targets in Escherichia coli. Annu. Rev. Genet 1996;30:465–506. [PubMed: 8982462]
2. Jenal U, Hengge-Aronis R. Regulation by proteolysis in bacterial cells. Curr. Opin. Microbiol 2003;6:163–72. [PubMed: 12732307]
3. Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature 2003;426:895–899. [PubMed: 14685250]
4. Reinstein E, Ciechanover A. Narrative review: protein degradation and human diseases: the ubiquitin connection. Ann. Intern. Med 2006;145:676–684. [PubMed: 17088581]
5. Breusing N, Grune T. Regulation of proteasome-mediated protein degradation during oxidative stress and aging. Biol. Chem 2008;389:203–209. [PubMed: 18208355]
6. Powers ET, Morimoto RI, Dillin A, Kelly JW, Balch WE. Biological and chemical approaches to diseases of proteostasis deficiency. Annu. Rev. Biochem 2009;78:959–991. [PubMed: 19298183]
7. Yu AY, Houry WA. ClpP: a distinctive family of cylindrical energy-dependent serine proteases. FEBS Lett 2007;581:3749–3757. [PubMed: 17499722]
8. Striebel F, Kress W, Weber-Ban E. Controlled destruction: AAA+ ATPases in protein degradation from bacteria to eukaryotes. Curr. Opin. Struct. Biol 2009;19:209–217. [PubMed: 19362814]
9. Sauer RT, Bolon DN, Burton BM, Burton RE, Flynn JM, Grant RA, Hersch GL, Joshi SA, Kenniston JA, Levchenko I, Neher SB, Oakes ES, Siddiqui SM, Wah DA, Baker TA. Sculpting the proteome with AAA(+) proteases and disassembly machines. Cell 2004;119:9–18. [PubMed: 15454077]
10. Maurizi MR, Clark WP, Katayama Y, Rudikoff S, Pumphrey J, Bowers B, Gottesman S. Sequence and structure of ClpP, the proteolytic component of the ATP-dependent Clp protease of Escherichia coli. J. Biol. Chem 1990;265:12536–12545. [PubMed: 21972725]
11. Wang J, Hartling JA, Flanagan JM. The structure of ClpP at 2.3 Å resolution suggests a model for ATP-dependent proteolysis. Cell 1997;91:447–456. [PubMed: 9390554]

12. Thompson MW, Maurizi MR. Activity and specificity of *Escherichia coli* ClpAP protease in cleaving model peptide substrates. J. Biol. Chem 1994;269:18201–18208. [PubMed: 8027081]

13. Thompson MW, Singh SK, Maurizi MR. Processive degradation of proteins by the ATP-dependent Clp protease from *Escherichia coli*. Requirement for the multiple array of active sites in ClpP but not ATP hydrolysis. J. Biol. Chem 1994;269:18209–18215. [PubMed: 8027082]

14. Brotz-Oesterhelt H, Beyer D, Kroll HP, Endermann R, Ladel C, Schroeder W, Hinzen B, Raddatz S, Paulsen H, Henninger K, Bandow JE, Sahl HG, Labischinski H. Dysregulation of bacterial proteolytic machinery by a new class of antibiotics. Nat. Med 2005;11:1082–1087. [PubMed: 16200071]

15. Kirstein J, Hoffmann A, Lilie H, Schmidt R, Rubsam-Waigmann H, Brotz-Oesterhelt H, Mogk A, Turgay K. The antibiotic ADEP reprogrammes ClpP, switching it from a regulated to an uncontrolled protease. EMBO Mol. Med 2009;1:37–49. [PubMed: 20049702]

16. Yoo SJ, Seol JH, Shin DH, Rohrwild M, Kang MS, Tanaka K, Goldberg AL, Chung CH. Purification and characterization of the heat shock proteins HslV and HslU that form a new ATP-dependent protease in *Escherichia coli*. Mol. Cell 2000;103:633–643. [PubMed: 11067331]

17. Sousa MC, Trame CB, Tsuruta H, Wilbanks SM, Reddy VS, McKay DB. Crystal and solution structures of an HslUV protease-chaperone complex. J. Biol. Chem 2002;278:779–785. [PubMed: 12054822]

18. Bogyo M, McMaster JS, Gaczynska M, Tortorella D, Goldberg AL, Ploegh H. Covalent modification of the active site threonine of proteasomal beta subunits and the *Escherichia coli* homolog HslV by a new class of inhibitors. Proc. Natl. Acad. Sci. USA 1997;94:6629–6634. [PubMed: 9192616]

19. Groll M, Bajorek M, Kohler A, Moroder L, Rubin DM, Huber R, Glickman MH, Finley D. A gated channel into the proteasome core particle. Nat. Struct. Biol 2000;7:1062–1067. [PubMed: 11062564]

20. Rabl J, Smith DM, Yu Y, Chang SC, Goldberg AL, Cheng Y. Mechanism of gate opening in the 20S proteasome by the proteasomal ATPases. Mol. Cell 2008;30:360–368. [PubMed: 18471981]

21. Smith DM, Chang SC, Park S, Finley D, Cheng Y, Goldberg AL. Docking of the proteasomal ATPases' carboxyl termini in the 20S proteasome's alpha ring opens the gate for substrate entry. Mol. Cell 2007;27:731–744. [PubMed: 17803938]

22. Smith DM, Kafri G, Cheng Y, Ng D, Walz T, Goldberg AL. ATP binding to PAN or the 26S ATPases causes association with the 20S proteasome, gate opening, and translocation of unfolded proteins. Mol. Cell 2005;20:687–698. [PubMed: 16337593]

23. Gribun A, Kimber MS, Ching R, Sprangers R, Fiebig KM, Houry WA. The ClpP double ring tetradecameric protease exhibits plastic ring-ring interactions, and the N terminus of its subunits form flexible loops that are essential for ClpXP and ClpAP complex formation. J. Biol. Chem 2005;280:16185–16196. [PubMed: 15701650]

24. Bewley MC, Graziano V, Griffin K, Flanagan JM. The asymmetry in the mature amino-terminus of ClpP facilitates a local symmetry match in ClpAP and ClpXP complexes. J. Struct. Biol 2006;153:113–128. [PubMed: 16406682]

25. Ingvarsen H, Mate MJ, Hogbom M, Portnoi D, Benaroudj N, Alzari PM, Ortiz-Lombardia M, Unge T. Insights into the inter-ring plasticity of caseinolytic proteases from the X-ray structure of
**Mycobacterium tuberculosis** ClpP1. Acta Crystallogr. D Biol. Crystallogr 2007;63:249–259. [PubMed: 17242518]

31. Kim DY, Kim KK. The structural basis for the activation and peptide recognition of bacterial ClpP. J. Mol. Biol 2008;379:760–771. [PubMed: 18468623]

32. Grimaud R, Kessel M, Beuron F, Steven AC, Maurizi MR. Enzymatic and structural similarities between the *Escherichia coli* ATP-dependent proteases, ClpXP and ClpAP. J. Biol. Chem 1998;273:12476–12481. [PubMed: 9575205]

33. Maurizi MR, Singh SK, Thompson MW, Kessel M, Ginsburg A. Molecular properties of ClpAP protease of *Escherichia coli*: ATP-dependent association of ClpA and clpP. Biochemistry 1998;37:7778–7786. [PubMed: 9601038]

34. Bewley MC, Graziano V, Griffin K, Flanagan JM. Turned on for degradation: ATPase-independent degradation by ClpP. J. Struct. Biol 2009;165:118–125. [PubMed: 19038348]

35. Jennings LD, Bohon J, Chance MR, Licht S. The ClpP N-terminus coordinates substrate access with protease active site reactivity. Biochemistry 2008;47:11031–11040. [PubMed: 18816064]

36. Ortega J, Singh SK, Ishikawa T, Maurizi MR, Steven AC. Visualization of substrate binding and translocation by the ATP-dependent protease, ClpXP. Mol. Cell 2000;6:1515–1521. [PubMed: 11163224]

37. Kessel M, Maurizi MR, Kim B, Kocsis E, Trus BL, Singh SK, Steven AC. Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26 S proteosome. J. Mol. Biol 1995;250:587–594. [PubMed: 7623377]

38. Kim YI, Levchenko I, Fraczkowska K, Woodruff RV, Sauer RT, Baker TA. Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase. Nat. Struct. Biol 2001;8:230–233. [PubMed: 11224567]

39. Singh SK, Rozycki J, Ortega J, Ishikawa T, Lo J, Steven AC, Maurizi MR. Functional domains of the ClpA and ClpX molecular chaperones identified by limited proteolysis and deletion analysis. J. Biol. Chem 2001;276:29420–29429. [PubMed: 11346657]

40. Joshi SA, Hersch GL, Baker TA, Sauer RT. Communication between ClpX and ClpP during substrate processing and degradation. Nat. Struct. Mol. Biol 2004;11:404–411. [PubMed: 15064753]

41. Martin A, Baker TA, Sauer RT. Distinct static and dynamic interactions control ATPase-peptidase communication in a AAA+ protease. Mol. Cell 2007;27:41–52. [PubMed: 17612489]

42. Liu Y, Patricelli MP, Cravatt BF. Activity-based protein profiling: the serine hydrolases. Proc. Natl. Acad. Sci. USA 1999;96:14694–14699. [PubMed: 10611275]

43. Burton RE, Baker TA, Sauer RT. Energy-dependent degradation: Linkage between ClpX-catalyzed nucleotide hydrolysis and protein-substrate processing. Protein Sci 2003;12:893–902. [PubMed: 12717012]

44. Martin A, Baker TA, Sauer RT. Protein unfolding by a AAA+ protease is dependent on ATP-hydrolysis rates and substrate energy landscapes. Nat. Struct. Mol. Biol 2008b;15:139–145. [PubMed: 18223658]

45. Hersch GL, Burton RE, Bolon DN, Baker TA, Sauer RT. Asymmetric interactions of ATP with the AAA+ ClpX6 unfoldase: allosteric control of a protein machine. Cell 2005;121:1017–10127. [PubMed: 15989952]

46. Kim YI, Burton RE, Burton BM, Sauer RT, Baker TA. Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. Mol. Cell 2000;5:639–648. [PubMed: 10882100]

47. Siddiqui SM, Sauer RT, Baker TA. Role of the processing pore of the ClpX AAA+ ATPase in the recognition and engagement of specific protein substrates. Genes Dev 2004;18:369–374. [PubMed: 15004005]

48. Barkow SR, Levchenko I, Baker TA, Sauer RT. Polypeptide translocation by the AAA+ ClpXP protease machine. Chem. Biol 2009;16:605–612. [PubMed: 19549599]

49. Flynn JM, Levchenko I, Seidel M, Wickner SH, Sauer RT, Baker TA. Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. Proc. Natl. Acad. Sci. USA 2001;98:10584–10589. [PubMed: 11535833]

50. Levchenko I, Grant RA, Wah DA, Sauer RT, Baker TA. Structure of a delivery protein for an AAA + protease in complex with a peptide degradation tag. Mol. Cell 2003;12:365–372. [PubMed: 14536076]
51. Song HK, Eck MJ. Structural basis of degradation signal recognition by SspB, a specificity-enhancing factor for the ClpXP proteolytic machine. Mol. Cell 2003;12:75–86. [PubMed: 12887894]

52. Kenniston JA, Baker TA, Fernandez JM, Sauer RT. Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine. Cell 2003;114:511–520. [PubMed: 12941278]

53. Gottesman S, Clark WP, Maurizi MR. The ATP-dependent Clp protease of *Escherichia coli*. Sequence of clpA and identification of a Clp-specific substrate. J. Biol. Chem 1990;265:7886–7893. [PubMed: 2186030]

54. Singh SK, Grimaud R, Hoskins JR, Wickner S, Maurizi MR. Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP. Proc. Natl. Acad. Sci. USA 2000;97:8898–8903. [PubMed: 10922052]

55. Gottesman S, Clark WP, de Crecy-Lagard V, Maurizi MR. ClpX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*. Sequence and in vivo activities. J. Biol. Chem 1993;268:22618–22626. [PubMed: 8226770]

56. Farrell CM, Grossman AD, Sauer RT. Cytoplasmic degradation of ssrA-tagged proteins. Mol. Microbiol 2005;57:1750–1761. [PubMed: 16135238]

57. Martin A, Baker TA, Sauer RT. Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines. Nature 2005;437:1115–1120. [PubMed: 16237435]

58. Burton RE, Siddiqui SM, Kim YI, Baker TA, Sauer RT. Effects of protein stability and structure on substrate processing by the ClpXP unfolding and degradation machine. EMBO J 2001;20:3092–3100. [PubMed: 11406586]
Fig. 1.
Changes in ClpP activity in response to ClpX binding. (A) Rates of ClpP_{14} (0.5 μM) reactivity with the covalent active-site inhibitor rhodamine-FP (200 μM) with or without ClpX_{6} (1 μM) and ATPγS/ADP (5 mM). The inset gel shows the fluorescence of rhodamine-FP modified ClpP as a function of time after addition of the inhibitor. The graph represents quantification of band intensities from multiple experiments. Values are averages (n=3) ± 1 standard deviation (SD). (B) Rates of ClpP_{14} (0.3 μM) cleavage of sub-\textit{K}_{M} concentrations of a dipeptide, a decapeptide, and an icosapeptide were determined in the presence or absence of ClpX (0.5 μM hexamer plus 1 mM ATPγS) and were normalized to the rate observed for ClpP alone. Values are averages (n=3) ± 1 SD. (C) Rates of ClpP_{14} (0.3 μM) cleavage of the decapeptide (15 μM) were determined in the presence of wild-type ClpX or the ATPase defective ClpX^{E185Q} mutant (0.5 μM hexamer) and different nucleotides (1 mM). Values are averages (n=3) ± 1 SD. (D) ClpX•ATPγS stimulation of the rate of ClpP peptide cleavage or active-site modification (from the experiments in panels A and B) is plotted as a function of the molecular weight of the peptide substrate or the active-site inhibitor. The solid line is a fit to a second-order polynomial function but has no theoretical significance.
Fig. 2.
Activity of ClpP variants with mutations in the N-terminal channel loops. (A) Side view of the crystal structure of ClpP (pdb code 1YG6) showing the location of the N-terminal channel loops (residues 1–18) in blue and the Ser-His-Asp catalytic triad in red. (B) Rates of decapeptide (15 μM) cleavage by wild-type ClpP and the channel variants (0.3 μM). The channel-loop sequence for each mutant is shown. Values are averages (n=3) ± 1 SD. (C) Rates of succinyl-LY-AMC (50 μM) cleavage by ClpP and the channel variants (0.1 μM). Values are averages (n=3) ± 1 SD. (D) Rates of ClpXP degradation of GFP-ssrA (5 μM). Reactions contained ClpX (0.1 μM hexamer) and different quantities of wild-type ClpP or the channel variants. Solid lines are fits to a hyperbolic function. (E) Non-additive effects of ClpX (6.8
μM hexamer plus 1 mM ATPγS) and the AQTSAGAA\textsuperscript{15} channel mutations on ClpP (0.3 μM tetradecamer) cleavage of the decapeptide (15 μM). Values are averages (n=3) ± 1 SD.
Fig. 3.
Degradation of peptides containing an N-terminal Abz-FAPHMALVPY\textsuperscript{NO2}, a guest region, and a C-terminal KKAANDENYALAA (ssrA tag underlined). (A) Rates of ClpXP degradation of a 33 residue ssrA-tagged peptide (8 μM) with a [YG]5 guest region. Each reaction contained ClpP\textsubscript{14} or mutant variants (0.15 μM), SspB (8 μM), and different quantities of wild-type ClpX. Solid lines are fits to the Hill equation. (B) Rates of ClpX•ClpP or ClpX•ClpP-AQTSAGAA\textsubscript{15} of ssrA-tagged peptide substrates with different guest regions. The rates shown were corrected by subtracting background cleavage by ClpP or ClpP-AQTSAGAA\textsubscript{15} only. Values are an average of two determinations. Guest regions: [E\textsubscript{10}], 10 glutamic acids; [R\textsubscript{10}], 10 arginines; [K\textsubscript{10}], 10 lysines; [Q\textsubscript{10}], 10 glutamines, [A\textsubscript{6}], six alanines; [G\textsubscript{4}], four glycines; [P\textsubscript{5}], five prolines; [FG]\textsubscript{5}, five phenylalanine-glycine repeats; and [YG\textsubscript{5}], five tyrosine-glycine repeats. All reactions contained 8 μM peptide, 8 μM SspB, 0.15 μM ClpX\textsubscript{6}, and 4 μM ClpP\textsubscript{14}. 
Fig. 4.
Helix A plays a role in regulating peptide degradation. (A) Structure of a ClpP subunit (pdb code 1YG6) showing residues 1–18 of the N-terminal channel loop (blue), helix A (green), and the catalytic triad (red). (B) Pro4, Val6, and Ile19 from one subunit pack in a hydrophobic cluster with residues Leu24 and Phe49 from a neighboring ClpP subunit. (C) Rates of decapeptide (15 μM) degradation for wild-type ClpP14 and helix-A mutants (0.3 μM). (D) Decapeptide cleavage by Ile19 mutants (conditions as in panel C). Values shown in panels C and D are averages (n=3) ± 1 SD.
Fig. 5. Properties of channel-loop and helix-A mutants. (A) Analysis by SDS-PAGE of time courses of digestion (30 °C) of wild-type ClpP_{14} or variants (0.7 μM) with trypsin (0.5 μM). The initial shift in mobility observed for all proteins is caused by cleavage of the C-terminal His_{6} tag. (B) Rates of decapetide (15 μM) cleavage by single and double ClpP mutants (0.3 μM tetradecamer) containing the GGGGGGGG^{15} channel-loop substitution and/or the L24A helix-A mutation. Values are averages (n=3) ± 1 SD. (C) Degradation of the carboxymethylated ^{35}S-titin-I27-ssrA protein (5 μM) by wild-type ClpP_{14} or the I19A mutant (1 μM). Values are averages (n=2).
Fig. 6.
Interaction of ClpP variants with ClpX or ClpA. (A) Rates of degradation of GFP-ssrA (5 μM) were measured as a function of the concentration of ClpP or helix-A mutants in the presence of a covalently linked ClpX-ΔN trimer (100 nM pseudo-hexamer equivalents). The solid lines are fits to a hyperbolic function. (B) Rates of GFP-ssrA (5 μM) were measured as a function of the concentration of ClpP or helix-A mutants in the presence of ClpA (50 nM hexamer). The solid lines are expected for a 1:1 binding interaction with a $K_D$ of 10 nM.