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Asymmetric Nucleotide Transactions of the HslUV Protease

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ATP binding and hydrolysis are critical for protein degradation by HslUV, a AAA^+ machine containing one or two $HslU_6$ ATPases and the $HslV_{12}$ peptidase. Although each HslU homohexamer has six potential ATP-binding sites, we show that only three or four ATP molecules bind at saturation and present evidence for three functional subunit classes. These results imply that only a subset of HslU and HslUV crystal structures represents functional enzyme conformations. Our results support an asymmetric mechanism of ATP binding and hydrolysis, and suggest that molecular contacts between HslU and HslV vary dynamically throughout the ATPase cycle. Nucleotide binding controls HslUV assembly and activity. Binding of a single ATP allows HslU to bind HslV, whereas additional ATPs must bind HslU to support substrate recognition and to activate ATP hydrolysis, which powers substrate unfolding and translocation. Thus, a simple thermodynamic hierarchy ensures that substrates bind to functional HslUV complexes, that ATP hydrolysis is efficiently coupled to protein degradation, and that working HslUV does not dissociate, allowing highly processive degradation.

Keywords: AAA^+ protease; energy-dependent degradation; protein turnover; enzyme regulation; peptidase activation

Introduction

ATP fuels the operation of most molecular machines, including energy-dependent intracellular proteases. ATP-dependent proteases typically consist of a barrel-shaped peptidase, with the active sites for peptide-bond cleavage sequestered in an aqueous internal chamber, and associated hexameric AAA^+ ATPases that recognize specific protein substrates, unfold these molecules, and then translocate the denatured polypeptide into the peptidase chamber for degradation.[1,2] AAA^+ and related P-loop ATPases also operate in many other biological processes in which mechanical work is performed on macromolecules.[3,4]

HslUV is an ATP-dependent protease that is present in roughly 60% of eubacteria and many eukaryotic lineages.[5] Functional HslUV enzymes are produced by the binding of one or two AAA^+ $HslU_6$ hexamers to $HslV_{12}$, a double-ring dodecameric peptidase that shares an N-terminal active-site threonine and extensive structural homology with the β-subunits of the eukaryotic proteasome.[6–8] Importantly, crystal structures of $HslU_6$, $HslV_{12}$, $HslU_6{\cdot}HslV_{12}$, and $HslU_6{\cdot}HslV_{12}{\cdot}HslU_6$ (Fig. 1a) have been solved.[8,10–18] Moreover, many HslU structures differ in conformation, apparently as a consequence of differences in bound nucleotides, providing clues about potential nucleotide-dependent movements that may drive protein unfolding and translocation. Indeed, HslUV is the only ATP-dependent protease for which the entire structure is known. However, several issues have hampered further understanding of this enzyme. First, structures of HslU hexamers with six, four, or three bound nucleotides have been solved. Which of these structures are functionally relevant is unclear. Second, whether the nucleotide bound in different HslU crystal structures is ADP or ATP has been controversial.[19] Third, there is little available quantitative information about nucleotide binding to HslU in solution or about the functional consequences of this binding on HslU recognition of HslV and/or protein substrates.

ClpX is a hexameric ATPase that shares about 50% sequence homology and substantial structural homo-

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Abbreviations used: ITC, isothermal titration calorimetry; mant, *N*-methylanthraniloyl; SPR, surface plasmon resonance.

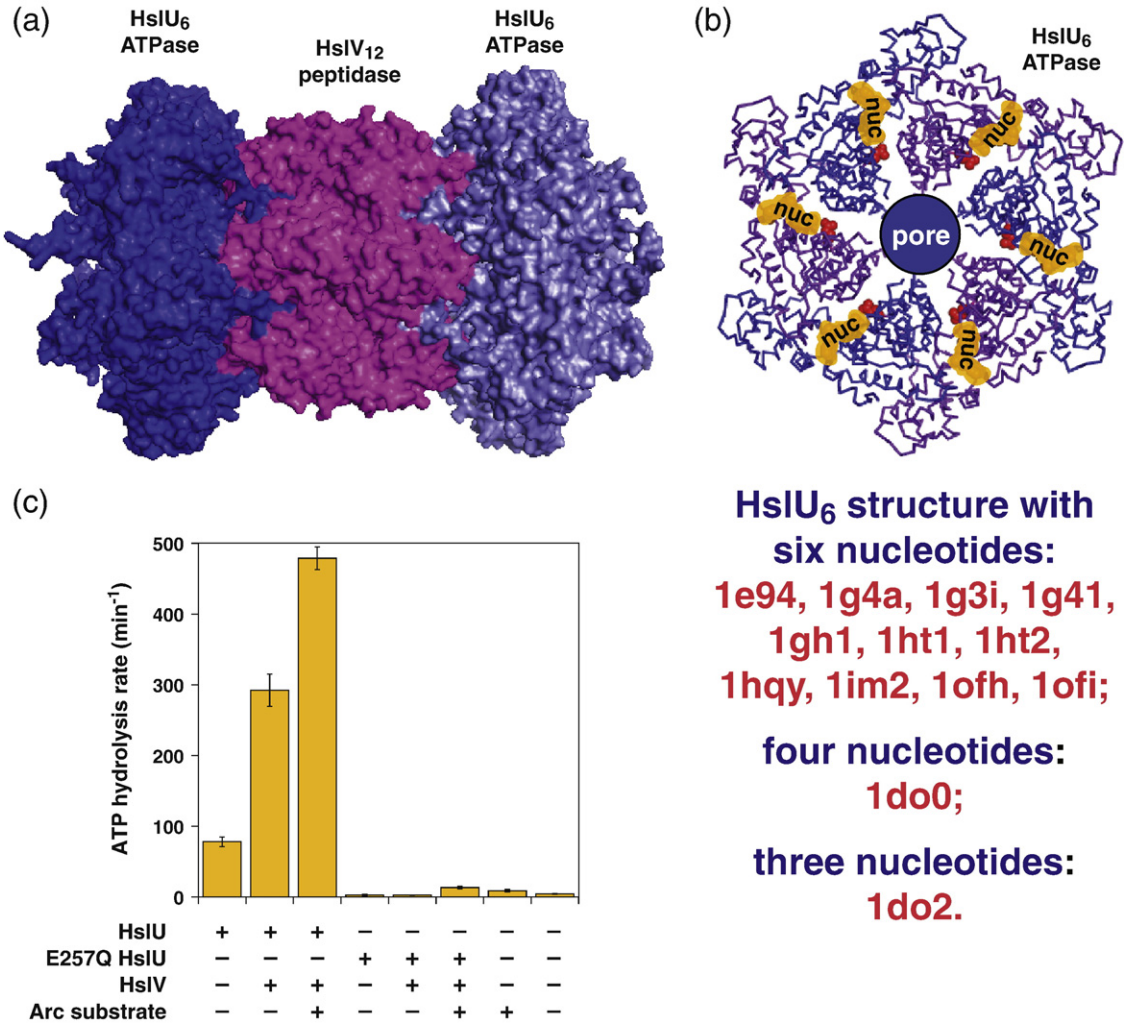

Fig. 1. HslU. (a) Two $HslU_6$ ATPases (blue) can assemble with the $HslV_{12}$ peptidase (magenta). (b) HslU hexamers have six potential nucleotide-binding sites, located at domain and subunit interfaces. From three to six nucleotides bind $HslU_6$ in different crystal structures. (c) Hydrolysis of ATP (2.5 mM) was measured at 37 °C in PD buffer in the presence or in the absence of HslU or E257Q HslU (0.3 μM hexamer), HslV (0.8 μM dodecamer), and Arc-IA37-st11-titin-ssrA substrate[9] (10 μM). Reaction components were preincubated with 10 μM ATPγS to promote HslU or HslUV association prior to addition of ATP.

logy with HslU.[3,20] Under conditions of ATP saturation, hexamers of ClpX bind only three or four molecules of ATP.[21] In addition, single-chain ClpX hexamers are functional, with two subunits capable of hydrolyzing ATP, two hydrolytically inactive subunits capable of assuming an ATP-bound conformation, and two subunits mimicking ATP-free wild-type subunits.[22] Studies of single-chain ClpX variants also suggest that hydrolysis is probabilistic, in the sense that different subunits hydrolyze ATP in a sequential—but not necessarily ordered—fashion. If HslU and ClpX function in the same manner, then HslU hexamers would also operate sequentially, always containing a mixture of nucleotide-bound and nucleotide-free subunits. However, ClpX partners with ClpP, a peptidase that shares no structural homology with HslV;[23] thus, it is not obvious that ClpX and HslU will operate by similar mechanisms. Indeed, structural studies of several other hexameric ATPases have been interpreted as evidence that these enzymes cycle between states that are fully ATP-bound and states that are fully ADP-bound.[24–26] Moreover, most HslU and HslUV crystal structures have six bound ATPs or ADPs (Fig. 1b), apparently supporting a symmetric or a concerted mechanism of ATP hydrolysis.

Here, we demonstrate that only three or four molecules of ATP bind to the HslU homohexamer at saturation. Thus, some of the six potential binding sites in the hexamer remain empty. ATP binds the sites that can be filled with comparable affinities, although the kinetics of nucleotide dissociation reveals the existence of two classes of binding sites. Our results support an ATPase model in which HslU subunits function asymmetrically and highlight structures with three or four bound nucleotides as being most relevant for understanding HslU function. Importantly, a single bound ATP supports HslV binding by the HslU hexamer but does not allow ATP hydrolysis; multiple ATPs must be bound to