

Chapter 1

Machines of Destruction – AAA+ Proteases and the Adaptors That Control Them

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Abstract Bacteria are frequently exposed to changes in environmental conditions, such as fluctuations in temperature, pH or the availability of nutrients. These assaults can be detrimental to cell as they often result in a proteotoxic stress, which can cause the accumulation of unfolded proteins. In order to restore a productive folding environment in the cell, bacteria have evolved a network of proteins, known as the protein quality control (PQC) network, which is composed of both chaperones and AAA+ proteases. These AAA+ proteases form a major part of this PQC network, as they are responsible for the removal of unwanted and damaged proteins. They also play an important role in the turnover of specific regulatory or tagged proteins. In this review, we describe the general features of an AAA+ protease, and using two of the best-characterised AAA+ proteases in *Escherichia coli* (ClpAP and ClpXP) as a model for all AAA+ proteases, we provide a detailed mechanistic description of how these machines work. Specifically, the review examines the physiological role of these machines, as well as the substrates and the adaptor proteins that modulate their substrate specificity.

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General Introduction

The bacterial cytosol is a complex mixture of macromolecules (proteins, DNA and RNA), which perform a variety of different functions. Given that proteins play a central role in many of these important cellular tasks, their correct maintenance within the cell is critical for cellular viability, not only under normal cellular conditions but also under conditions of stress. As such, a bacterial cell contains a network of molecular chaperones and proteases (often referred to as the protein quality control (PQC) network) dedicated to maintaining homeostasis of protein folding. Chaperones function to protect functional proteins against unfolding and to refold misfolded and aggregated species. The role of proteases is to remove unwanted and hopelessly damaged proteins.

In the bacterial cytosol, protein degradation is performed mainly by a number of different ATP-dependent proteolytic machines. In general these machines are composed of two components, a peptidase and an unfoldase. Invariably, the unfoldase is a member of the AAA+(ATPase associated with diverse cellular activities) superfamily and as such these molecular machines are commonly referred to as AAA+ proteases [1]. In Gram-negative bacteria, such as *Escherichia coli* there are generally five different AAA+ proteases (ClpAP, ClpXP, HslUV, Lon (also referred to as LonA) and FtsH). In contrast most Gram-positive bacteria, such as *Bacillus subtilis*, contain up to seven different AAA+ protease (ClpCP, ClpEP, ClpXP, HslUV (CodXW), LonA, LonB and FtsH). Interestingly, in bacteria belonging to the Actinobacteria and Nitrospira phyla (e.g. in *Mycobacterium tuberculosis* (*Mtb*)) one or more of these AAA+ proteolytic machines is replaced by the proteasome (for a detailed review of this AAA+ machine, and its physiological role in *Mtb* please refer to Darwin and colleagues [2]). Regardless of their origin, these machines can be divided into two broad groups; those that contain the unfoldase and peptidase components on separate polypeptides (e.g. ClpAP, ClpCP, ClpEP, ClpXP and HslUV (CodXW)), and those that contain both components on a single polypeptide (e.g. LonA, LonB and FtsH) (see Fig. 1.1).

This review will focus on the “two-component” proteolytic machines, primarily those from *E. coli* (e.g. ClpAP and ClpXP), with a brief comparison to the equivalent machines (e.g. ClpCP and ClpXP) in the model Gram-positive bacterium, *B. subtilis*. However, for an extensive review on regulatory proteolysis in *B. subtilis* please refer to [3]. Likewise, for a detailed review on the “single polypeptide” proteases, i.e. Lon and FtsH please refer to [4] and [5], respectively.

Structure and Function of the “ClpP Containing” Proteases (ClpAP, ClpXP and ClpCP)

As mentioned above, bacteria contain a wide variety of different proteolytic machines, of which ClpXP is certainly the best-studied AAA+ protease [1]. ClpXP is known to play a number of critical roles in a wide variety of bacterial species, from the control of different stress response pathways in Gram-positive and Gram-negative

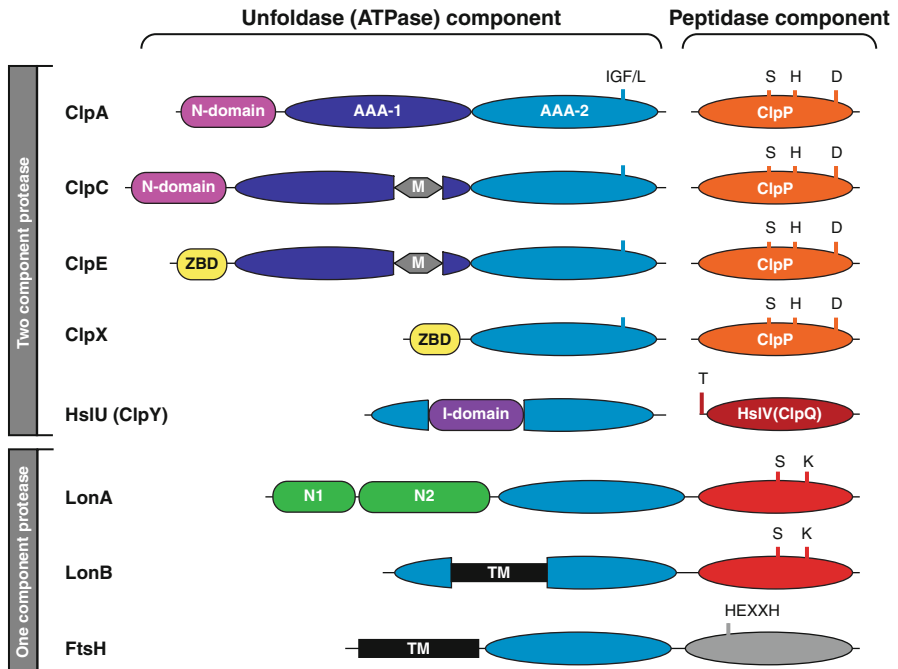


Fig. 1.1 Cartoon representation of the various different AAA+ proteases in bacteria. AAA+ proteases can be separated into two different groups. Two component proteases (e.g. ClpAP, ClpCP, ClpXP, ClpEP and HslUV) contain the unfoldase and peptidase components on separate polypeptides. One component proteases, contain the peptidase and the unfoldase on a single polypeptide (e.g. LonA, LonB and FtsH). The unfoldase component contains one or more AAA+ domains, responsible for ATP-dependent unfolding of the substrate. All unfoldase components also contain at least one accessory domains (e.g. ClpA and ClpC contain a conserved N-terminal domain (N-domain, pink), ClpC and ClpE contain a middle domain (M, grey), ClpE and ClpX contain a Zinc binding domain (ZBD, yellow), HslU contains an accessory domain inserted into the AAA+ domain (I-domain, purple), LonA contains two N-terminal domains unrelated to the N-domain of ClpA and ClpC (N1 and N2, green), while LonB and FtsH both contain a single transmembrane (TM) region), which serve various different functions (see main text for details). In the case of the ClpP-binding unfoldase components, the AAA-2 domain contains an IGF/L loop for interaction with ClpP. The protease components are responsible for cleavage of the unfolded substrate. In the case of ClpP, hydrolysis of the polypeptide is catalysed by the catalytic triad (S, H and D), while FtsH and HsIV contain either a conserved HEXXH motif or an N-terminal threonine (T) respectively, for peptide bond cleavage

bacteria (see [6, 7]) to the regulation of virulence through the degradation of key factors that control virulence (see [8]). ClpXP has also been shown to play an important role in regulating mitochondrial protein homeostasis (proteostasis) in eukaryotes such as worms [9, 10] and plants [11]. Surprisingly however, this proteolytic machine is absent from most fungi including, *Saccharomyces cerevisiae* [12, 13]. For a detailed review of about the role of these AAA+ proteases in regulating mitochondrial function please refer to [14]. Although the AAA+ proteases ClpAP and

ClpCP are not as widely conserved as ClpXP, these proteases do, nevertheless, control a number of key proteolytic/regulatory pathways in Gram-negative and Gram-positive bacteria, respectively. Interestingly, ClpCP also appears to play an important role in proteostasis within the plastid of plants (for a recent review see [15, 16]).

Although these machines recognise a variety of different substrates and regulate a range of different physiological processes, each machine shares a common architecture and a similar mode of action. All form barrel-shaped complexes in which the oligomeric AAA+ unfoldase is concentrically aligned with the oligomeric protease component as is best illustrated by the crystal structure of the HslUV complex [17, 18]. Interestingly, the unfoldase component may be located at either or both ends of the peptidase component to form single-headed (1:1) or double-headed (2:1) complexes, respectively. For the ClpAP protease, the symmetric double-headed complexes have been shown to be most efficient at processing substrates [19]. Regardless of whether the complexes are single- or double-headed, both oligomeric components (i.e. the unfoldase and the peptidase) generally exhibit a six-fold symmetry throughout the entire complex. However in the Clp protease complexes (e.g. ClpAP, ClpCP and ClpXP) the machines display a unique symmetry mismatch between the unfoldase and the peptidase. While the AAA+ unfoldase component (i.e. ClpA, ClpC and ClpX) like most AAA+ proteins studied to date, form hexameric ring-shaped oligomers, the peptidase (i.e. ClpP) is composed of two heptameric rings [20]. The two heptameric rings of ClpP stack back-to-back, encapsulating the catalytic (active site) residues of ClpP within a barrel shaped tetradecamer. This symmetry mismatch poses some interesting questions. How do these two rings (the hexameric unfoldase and the heptameric peptidase) interact to form a functional complex, and how many subunits are required for a functional interaction. Regardless of whether the protease complex is symmetric or asymmetric, all AAA+ proteases undergo three basic steps in order to degrade a substrate protein (see Fig. 1.2). In the first step, the substrate is recognised by the unfoldase, although in some cases substrate recognition may be facilitated by an adaptor protein (see later). In bacteria, substrates are usually recognised via short sequence specific motifs (termed degrons), which are often located at the N- or C-terminus of the substrate protein. Following recognition, the substrate is then unfolded in an ATP-dependent fashion (Fig. 1.2, step 2). The unfolded substrate is then translocated into the associated peptidase, where the polypeptide chain is hydrolysed into small peptide fragments (~3–8 amino acids long), which have been proposed to egress through the holes in the sidewall of the peptidase, although this method of egress remains somewhat controversial (Fig. 1.2, step 3) [21, 22].

The Peptidase ClpP

The ClpP peptidase is synthesized as a zymogen, containing a N-terminal propeptide [23], which is autocatalytically cleaved upon oligomerization, resulting in the formation of a proteolytically active oligomer. ClpP is a serine protease, composed of a

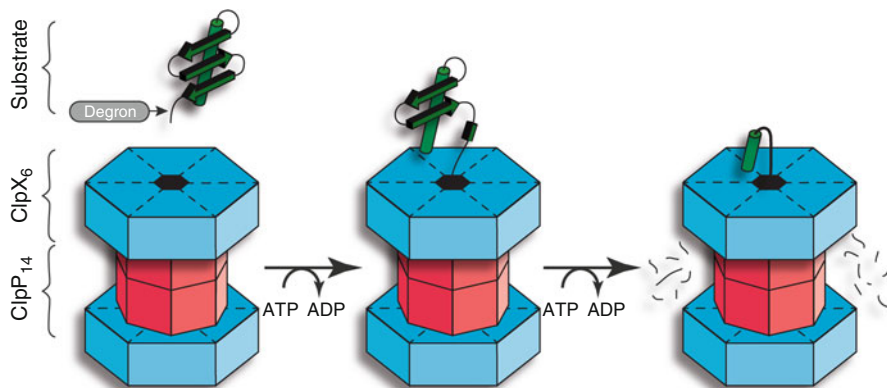


Fig. 1.2 Cartoon illustrating the main steps involved in substrate recognition and degradation by AAA+ proteases. The unfoldase (e.g. ClpX) forms a hexameric ring-shaped structure (blue) at one or both ends of the peptidase (e.g. ClpP), which forms two heptameric rings stacked back-to-back (red). The substrate (green) contains a degradation signal (degron) often located at the N- or C-terminus of the protein. The degron is recognised by the unfoldase and the substrate protein unfolded, in an ATP-dependent fashion, then translocated into the peptidase where the protein is cleaved into small peptide fragments, which diffuse through holes in the side-wall of the peptidase

Ser-His-Asp catalytic triad (Fig. 1.1), which exhibits chymotrypsin-like activity, that is, it cleaves peptide bonds mostly after non-polar residues [24, 25]. The active peptidase is a barrel-shaped oligomer composed of two heptameric rings, stacked back-to-back [20], that forms a degradation chamber in which the proteolytic active sites are sequestered away from cytosolic proteins (Fig. 1.3a). Each monomer of ClpP resembles a hatchet and consists of three subdomains: a handle, a globular head and a N-terminal loop. The heptameric ring is formed by the interaction of seven subunits through the head subdomain, and the tetradecamer is formed by the interaction of two heptameric rings through the handle subdomain (Fig. 1.3a). Entry into the catalytic chamber of this serine peptidase is restricted to a narrow entry portal (~ 10 Å) at both ends of the barrel-shaped complex. The N-terminal peptides of ClpP flank the axial pore and are proposed to act as a gate for entry into the proteolytic chamber. As a result of this narrow axial entry portal, folded proteins are excluded from entering the catalytic chamber, although small peptides and unfolded proteins can be degraded in an ATPase independent fashion, albeit unfolded proteins are degraded very slowly in the absence of the ATPase [26]. Importantly, the degradation of unfolded substrates can be accelerated by the addition of a cognate unfoldase (i.e. ClpX, ClpA or ClpC), which implies that entry into ClpP is gated and that this gated-entry can be activated by the unfoldase. Indeed, recent cryo-EM reconstructions have shown that binding of ClpA triggers a change in the N-terminal loops of ClpP, from a “down” conformation where they block entry to the catalytic chamber, to an “up” conformation which permits access to the chamber [27]. Consistent with a “gating” role for the N-terminal loops of ClpP, deletion of these loops was

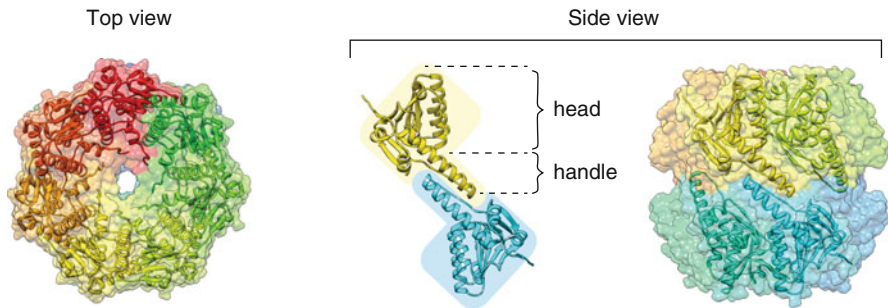
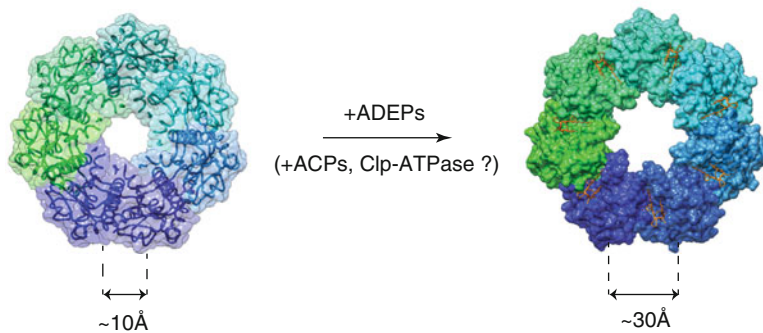
a *E. coli* ClpPb *B. subtilis* ClpP

Fig. 1.3 Oligomeric structure of ClpP. (a) ClpP (PDB: 1TYF) forms two heptameric ring-shaped oligomers (*Top view*) stacked back-to-back (*Side view*) to create a barrel-shaped oligomer. Interactions between adjacent head subdomains drive oligomerisation of the seven-membered ring, while interactions between the handle subdomain of two heptamers are responsible for formation of the tetradecamer. (b) In the absence of the unfoldase, the entry portal into the catalytic chamber of ClpP (PDB: 3KTH) is narrow ($\sim 10\text{\AA}$), in the presence of chemical activators of ClpP (i.e. ADEPs, ACPs and potentially the unfoldase), the entry portal into the catalytic chamber of ClpP (PDB: 3KTI) is opened ($\sim 30\text{\AA}$)

shown to accelerate the degradation of short peptides [28]. The cognate AAA+ unfoldase also mediates the degradation of folded substrate proteins by actively unfolding and translocating the substrates through the axial pore and into the proteolytic chamber of ClpP. Indeed, it appears that the oligomeric structure of ClpP has been carefully designed to prevent widespread and indiscriminate degradation of cellular proteins by regulating substrate access to its proteolytic chamber. Consistent with this idea, several recent studies have identified a series of novel antibiotics (e.g. acyldepsipeptides (ADEPs) and ACPs) that activate ClpP (in the absence of its cognate unfoldase) for unregulated protein degradation [29–34]. This activation of ClpP

results in the unregulated degradation of nascent polypeptides and unfolded proteins in the cell [34], and in a recent study ADEP was shown to inhibit cell division of Gram positive bacteria, through the ClpP-mediated degradation of FtsZ, a key protein required for septum formation [35].

Based on a series of biochemical and structural studies, these chemical activators of ClpP dock into a hydrophobic pocket located on the surface of ClpP (Fig. 1.3b). Firstly, and most importantly, ADEP binding to this hydrophobic pocket results in opening of the ClpP pore (from ~ 10 Å in the absence of ADEP to ~ 21 – 27 Å in the presence of different forms of ADEP). This “gated-opening” of the ClpP pore, is proposed to be sufficient to allow entry of unfolded proteins into the proteolytic chamber of ClpP (where the catalytic residues are located) and possibly the primary reason for degradation of unfolded substrates. Interestingly, in the case of *B. subtilis* ClpP, ADEP not only triggers opening of the pore, but also triggers oligomerisation of ClpP from free “inactive” monomers to “active” tetradecamers [32], a step that is normally controlled by the cognate unfoldase, ClpC [36]. Similarly, ADEP activation of human ClpP for unregulated degradation is also likely to result from assembly of the ClpP tetradecamer [37] a process that normally requires the assistance of ClpX [38]. As a consequence, ADEP also appears to be a competitive inhibitor of unfoldase binding to ClpP, preventing the regulated degradation of substrates that would normally be delivered to ClpP by the unfoldase component [32]. As such, the ADEP-bound conformation of ClpP has been proposed to mimic the unfoldase-bound conformation of ClpP. Surprisingly, binding of ClpA to ClpP, as measured from sections of the ClpAP cryo-EM structure, appears to have little effect on the size of the ClpP pore (diameter ~ 12 Å) [27] and hence it has been suggested that the size of the pore may vary with translocation of different substrates [39]. Nevertheless, it remains to be seen, if an ordered arrangement of the N-terminal loops on ClpP (as observed in the *B. subtilis* ClpP-ADEP structure) or a disorder arrangement of the N-terminal loops of ClpP (as observed in the *E. coli* ClpP-ADEP complex) resembles the unfoldase bound complex.

The Unfoldase Components (ClpX/ClpA/ClpC)

In *E. coli*, ClpP forms proteolytic complexes with both ClpA and ClpX, while in *B. subtilis*, ClpP associates with three different unfoldases, ClpC, ClpX and ClpE [3]. Although the overall architecture of the different unfoldase components is similar, each unfoldase contains a unique organisation. While ClpA, ClpC and ClpE each contain two AAA+ domains, ClpX only contains a single AAA+ domain (Fig. 1.1). Regardless of the number of AAA+ domains present, each unfoldase contains one or more accessory domains. In the cases of ClpA and ClpX, a single accessory domain is located at the N-terminus of the protein, while both ClpC and ClpE contain two accessory domains, one at the N-terminus of the protein and the other located between the two AAA+ domains, termed the middle or M-domain (Fig. 1.1). In general, these accessory domains are required for the binding of substrates and/

or adaptor proteins. In the case of ClpA, the N-terminal domain is essential for docking of the adaptor protein ClpS [40–42] but also required for the recognition, and hence degradation of some substrates [43, 44]. Similarly, the N-terminal domain of *B. subtilis* ClpC is essential for the ClpP-mediated degradation of most substrates [45, 46]. However in this case, the N-domain is thought not to be directly involved in substrate recognition but rather plays a crucial role in binding adaptor proteins (i.e. MecA and McsB), which are required for ClpC oligomerisation and/or substrate delivery [36, 47, 48]. Interestingly in the case of *B. subtilis* ClpC, the second accessory domain (the M-domain) located between the two AAA+ domains, also plays an important role in the recognition of adaptor protein, however the details of substrate delivery by these adaptor proteins is currently unknown [36, 46–48]. For further details regarding the mechanism of action of ClpCP please refer to [3].

In the case of ClpX (and ClpE from Gram-positive bacteria) the N-terminal accessory domain (residues 1–60 in *E. coli* ClpX) is a C4-type Zinc binding domain (ZBD), which contains four Cysteine residues that coordinate a single Zn atom. In *E. coli* ClpX, this domain forms a very stable dimer [49], and is responsible for the recognition of several substrates (such as λ O and MuA) but not SsrA-tagged proteins [50–52]. This domain is also essential for the recognition of the adaptor proteins, SspB [50, 52, 53] and UmuD [54], discussed in more detail later.

Given that *E. coli* ClpX is, by far the most extensively characterised Clp-ATPase, this section will focus primarily on the structure and function of ClpX. However, many of the features described here for the AAA+ domain of ClpX are likely to be generally applicable to most AAA+ proteases. At a structural level, the AAA+ domain (~200–250 a.a.) is composed of two subdomains – a large N-terminal subdomain, which forms an α/β wedge-shaped Rossmann fold and a small C-terminal subdomain, which forms a α -helical lid across the nucleotide-binding site [55, 56]. ATP is bound in a cleft between the large and small subdomain of a single subunit and the large subdomain of the adjacent subunit. As such, these interactions provide much of the driving force for formation of the hexamer. To date, several highly conserved sequence motifs have been identified within the AAA+ domain, each of which is responsible for a specific function [57]. The Walker A motif (GXXXXGK [T/S], where X=any amino acid) is required for ATP binding and facilitates oligomerization of the protein into ring-shaped hexamers. The Walker B motif (hhhhDE, where h=any hydrophobic amino acid) is required for hydrolysis of bound ATP and hence drives conformational changes in the protein, mediating substrate binding and translocation. The central pore of the hexamer is comprised of several important motifs and loops (e.g. the pore-1 loop) involved in substrate binding [58–61]. The Sensor 1 and 2 motifs, together with the arginine fingers, are proposed to couple the nucleotide-bound state of the oligomer with conformational changes in the subdomains, which through movement of the substrate-binding loops, results in substrate unfolding and translocation [55, 58]. Despite the broad sequence conservation of AAA+ domains, individual AAA+ domains appear to serve different functions in proteins that contain two or more AAA+ domains (i.e. ClpA or ClpC) [62]. For example, the first AAA+ domain (D1) in ClpA is crucial for oligomerisation while the second AAA+ domain (D2) is primarily responsible for ATP hydrolysis [63]. Interestingly,

variants of ClpA lacking ATPase activity in either D1 or D2, are only able to process substrates with “intermediate” or “low” local stability respectively, suggesting that each domain can function independently, at least to a limited extent [64]. However, the ATPase activity of both domains is required for the efficient processing of substrates with “high” local stability [64] indicating that both domains work together to unfold and translocate substrates into ClpP.

As viewed from the top (or ClpP distal face) of the unfoldase, the ClpX hexamer can be divided into six units, each of which was composed of a small AAA+ subdomain from one subunit with a large AAA+ subdomain of the adjacent subunit [55, 56]. Recently, it was shown that the structures of all six of these units were highly superimposable [55] and hence it was proposed that each unit forms a functional rigid body (Fig. 1.4a, b). Despite the high degree of structural similarity between each rigid body unit, the overall shape of the ClpX hexamer is asymmetric, which suggests that the angle of the hinge between the rigid body units (i.e. the angle between the large and the small subdomains within a single subunit of ClpX) varies. This difference in the angle between the rigid body units results in a different ability of each subunit to bind nucleotide. Based on this description, each subunit within the ClpX hexamer can be classified into one of two groups; type 1 subunits, which are able to bind nucleotide (referred to as L, for “loadable”), and type 2 subunits, which are unable to bind nucleotide (referred to as U, for “unloadable”). In the crystal structure of ClpX, the hexamer is composed of four L (or type 1) subunits and two U (or type 2) subunits arranged in the following manner, L-L-U-L-L-U (Fig. 1.4c). Therefore, given that ATP binding and hydrolysis is expected to stabilise the L conformation, while the release of ADP is predicted to result in a transition from the L to the U conformation, it is proposed that the ATPase activity of ClpX will promote domain rotations within a subunit that will propagate around the hexamer and drive transition of the other subunits, in a chain reaction. These ATPase-induced conformational changes are proposed to form an integral part of the mechanism for substrate translocation by ClpX into ClpP (see later).

The Unfoldase-Peptidase Complex

Given that the AAA+ unfoldase component (i.e. ClpX, ClpA or ClpC) is hexameric and the associated peptidase (e.g. ClpP) is formed by two heptameric rings, the resulting proteolytic machines, ClpXP (ClpAP and ClpCP), exhibit an asymmetry between the two components. This asymmetry, although not unique in biology, poses several interesting questions. How do the two components interact with one-another? How many of these features per hexamer (i.e. how many subunits) are required for formation of a functional complex? Not surprisingly, the formation of the complex is transient, and efficient interaction of the two components is dependent on nucleotide-bound state of the unfoldase. Specifically, formation of the ClpXP complex is only supported by ATP, ATP γ S (a slowly hydrolysable analogue of ATP) or a ClpX mutant that is defective in ATP hydrolysis [65]. In contrast, the complex

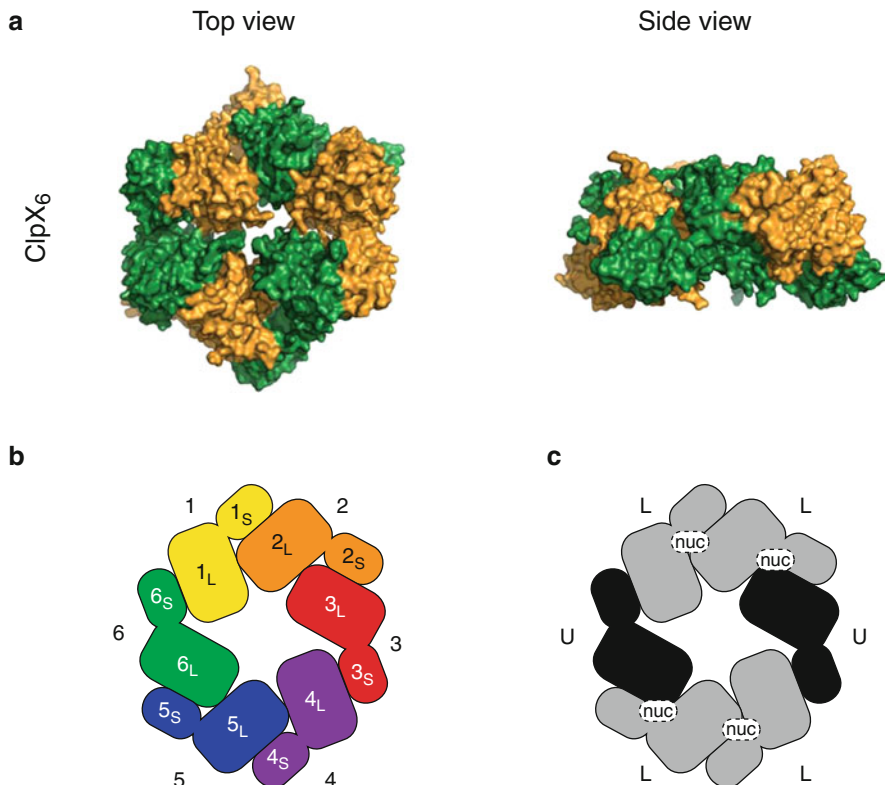


Fig. 1.4 Oligomeric structure of ClpX. In the presence of nucleotide, ClpX forms a hexameric *ring-shaped* oligomer. **(a)** Surface representation of the ClpX hexamer (PDB: 3HWS). **(b)** Cartoon, illustrating the asymmetric organisation of the ClpX hexamer. **(c)** The asymmetric organisation of the ClpX hexamer results from a differential binding of nucleotide (nuc) within the hexamer. Nucleotides are bound in a cleft formed by the large and small domain of one subunit and the large domain of the adjacent subunit. Depending on the orientation of the small and large domain within a subunit, a subunit can be classified into two types; loadable (L) which are able to bind nucleotide and unloadable (U) which are unable to bind nucleotide. The arrangement of these different subunit types, within the ring gives rise to an asymmetric appearance of the hexamer

dissociates in the presence of ADP or in the absence of nucleotide [66, 67]. This interaction, (i.e. between the two components), is mediated by two sets of contacts; one at the periphery of the interface and the other near the central pore. The peripheral contact occurs between a flexible loop on ClpX and a hydrophobic pocket on the surface of ClpP, and is important for a strong, nucleotide-independent interaction with ClpP. The flexible loop contains a conserved tripeptide motif ([L/I/V]-G-[F/L]) and as such is often referred to as the IGF/L-loop (Fig. 1.5a). This motif is unique to ClpP-binding unfoldases (i.e. ClpA, ClpC, ClpE and ClpX) and is essential for interaction with ClpP [68, 69]. Consistently, mutation of this motif dramatically reduces the affinity of ClpX to ClpP [67, 68]. The second contact

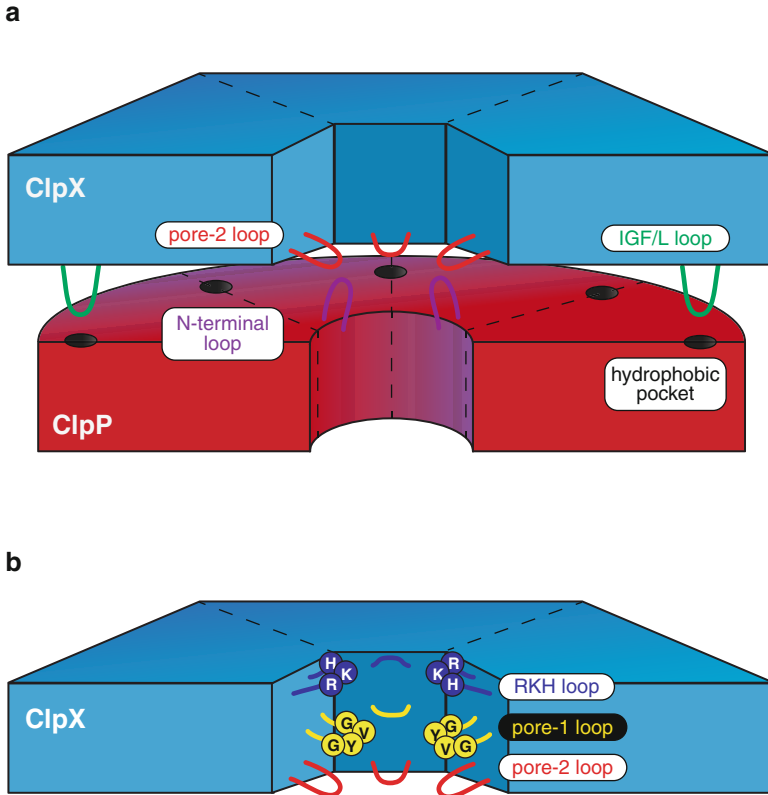


Fig. 1.5 ClpP-binding and substrate interaction is mediated by several loops and pockets. (a) Cut-away view of ClpX (blue), highlighting the important interactions that contribute to complex formation with ClpP (red). The IGF/L loops (green) on ClpX form a static interaction with the hydrophobic pocket on ClpP (black). ClpXP complex formation is modulated by the nucleotide state of ClpX, through a set of dynamic interactions, between pore-2 loops of ClpX (red) and the N-terminal loop of ClpP (purple). (b) The substrate is recognised and translocated through the pore via a set of conserved pore loops; RKH (blue), pore-1 (yellow) and pore-2 (red). These loops move up and down the pore of ClpX in a nucleotide-dependent fashion, thereby translocating the substrate into ClpP

occurs between two loops; one loop (termed the pore-2 loop) protrudes from the axial pore of ClpX, and interacts with the N-terminal loop of ClpP [21, 70, 71]. This interaction, between the two axial loops, appears to be highly dynamic and is dependent on the nucleotide-state of individual subunits of ClpX [71]. Although the ClpXP complex is asymmetric, both sets of loops (the IGF/L-loop, for docking into the hydrophobic pocket on ClpP and the two axial pore loops) appear to be flexible enough that contacts from each subunit of ClpX contribute to the interaction. Indeed loss of a single IGF-loop, within the ClpX hexamer, is sufficient to reduce ClpP binding and activity, while loss of more than one contact per hexamer completely abolishes ClpP binding [71].

Degradation Recognition Motifs (Degrons)

A bacterial cell is composed of thousands of different proteins, the concentration (or copy number) of which varies dramatically (from ~100 to 10^5 molecules per cell) [72]. Likewise, the concentration of each individual protein varies in response to changing environmental conditions or stress. As such, in order for the cell to maintain optimal function, not only under normal conditions but also under conditions of stress, the composition and active concentration of its proteins must be monitored and maintained. Hence it is important for the cell to specifically remove unwanted or damaged proteins from the cell when they are no longer required. To achieve this, bacterial proteases need to combine two seemingly incompatible properties, broad recognition of a range of different protein substrates, with a high degree of substrate specificity to prevent the recognition of properly folded or wanted cellular proteins.

A key feature of most, if not all, bacterial protein substrates is the presence of a specific amino acid motif, often referred to as a degradation tag or degron [73]. These degrons are generally located at the N- or C-terminus of the protein, although in some cases they are located internally. Although most degrons are intrinsic to the target protein, a handful of degrons (e.g. the SsrA tag and some N-end rule substrates) are not defined by the primary sequence of the protein, but rather are added (either co- or post-translationally) to the protein [74, 75]. Often, intrinsic degrons are only revealed (for recognition by the protease) following exposure of the protein to stress (e.g. heat-shock) or processing by an endoprotease [76–79]. This conditional recognition of a protein substrate is ideally suited to the controlled degradation of a key regulatory protein, and forms the basis of controlling several stress response pathways in bacteria (see [6]). In some cases however, a degron may be constitutively exposed under normal conditions, in order to maintain low levels of the protein (e.g. SigmaS) [80].

Trans-translation and the SsrA-Tag: A Specific Protein Tagging System in Bacteria

Messenger RNA molecules normally contain a stop codon at the 3' end of the transcript, which serves not only to signal the end of translation, but also triggers ribosome dissociation. In some cases however, as a result of truncation of the mRNA or errors during its transcription, the lack of a stop codon in the mRNA sequence caused “stalling” of protein synthesis [81–83]. To overcome this problem, bacteria possess a conserved mechanism, to restart translation and allow ribosome dissociation. This mechanism (illustrated in Fig. 1.6), often referred to as trans-translation, is sensed by an empty A-site and signalled by stalling of the translating ribosome [84]. This signal results in the recruitment of a specialised RNA molecule into the empty A-site of the ribosome. This RNA, encoded by *ssrA* (small stable RNA gene A) [85] has been termed a tmRNA as it functions both as a tRNA and as an mRNA [84, 86, 87]. The tRNA-like structure can be charged with alanine at its 3' end, while an extended

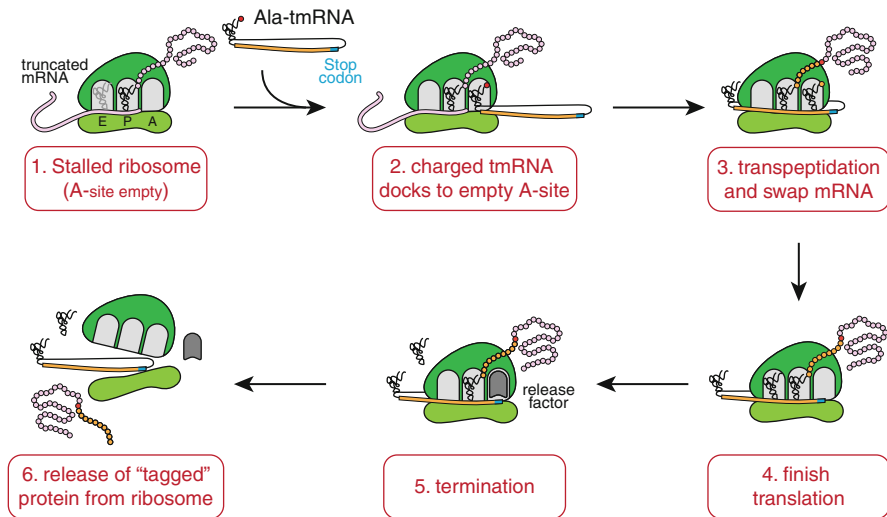


Fig. 1.6 Cartoon, illustrating the process of trans-translation. 1. Truncated mRNA (lacking a stop codon) cause “stalling” of the ribosome. 2. This “stalling” triggers binding of a tmRNA into the empty A-site of the ribosome. 3. Following a transpeptidation reaction, the truncated mRNA is replaced with the mRNA from the tmRNA and 4. translation proceeds, resulting in 5. correct termination of protein synthesis 6. rescuing the ribosome and releasing the “tagged” protein for targeted degradation by ClpXP

loop within the same RNA molecule encodes a short open reading frame (ten amino acids in *E. coli*) that ends in a stop codon. Following docking of the charged tmRNA into the empty A-site of the ribosome, the alanine is transferred to the nascent polypeptide and the open reading frame (encoded by the mRNA portion of the tmRNA) is translated. Noteworthy, trans-translation results in the attachment of a short C-terminal extension (termed the SsrA tag) to the incompletely synthesised protein.

Importantly, given that SsrA-tagged proteins are produced from aberrant or incomplete mRNA, it is unlikely that they will be able to fold. For this reason, interaction of SsrA-tagged proteins with chaperones is wasteful, as attempts to refold trans-translation products would be futile. Rather, SsrA-tagged proteins are rapidly degraded by proteases. In *E. coli*, the SsrA tag is 11 amino acids long (AANDENYALAA) and substrates tagged with the sequence are recognised by ClpXP, ClpAP and FtsH [81, 88–90]. Despite the fact that the SsrA tag is recognised by several different proteases *in vitro*, the *in vivo* degradation of these substrates is almost exclusively performed by ClpXP [81, 91].

Nevertheless, this tag has been used extensively as a model degron to study the function of both ClpXP and ClpAP. As such, it has proved to be a powerful research tool to study the mechanism of protein recognition and degradation by AAA+ proteases. A major advantage of the SsrA tag, as a research tool to study protein degradation, is that any protein can be converted into a ClpXP (or ClpAP) substrate, simply through the attachment of the SsrA tag to its C-terminus. This has permitted

a detailed mechanistic analysis of protein degradation using a range of different substrates with a variety of unique or desired features (i.e. green fluorescent protein (GFP) or the I27 domain of the human titin) to examine unfolding [92–95]. Likewise, it has also served as an excellent tool to study the mechanism of adaptor-mediated substrate delivery (see below).

Other ClpX Recognition Motifs

Apart from the specific recognition of the SsrA-tag, ClpX is also involved in the recognition of several other proteins, including a number of proteins involved in various stress response pathways. In order to determine the complete substrate-binding repertoire of *E. coli* ClpX, a mutant version of ClpP was used to capture the physiological substrates of ClpXP *in vivo* [96]. Using this approach, ~100 putative ClpXP substrate proteins were identified [96, 97]. Following verification of several of these proteins (either by *in vitro* or *in vivo* degradation assays) five different ClpX “recognition” motifs were proposed [96]. Of the five different “recognition” motifs, two were located near the C-terminus of the protein and three near the N-terminus of the protein (Fig. 1.7). While both classes of C-terminal motifs (C-motif 1 and 2, Fig. 1.7) shared homology with known ClpXP substrates (i.e. the SsrA-tag and MuA, respectively), only a single N-terminal motif (N-motif 1, Fig. 1.7) had been observed previously (i.e. λ O) [98].

Interestingly, the various degradation motifs appear to be recognised by different regions within the unfoldase. Some substrate classes (e.g. N-motif 1) strictly depend on interaction with the N-terminal domain, while other motif classes (e.g. C-motif 1, i.e. SsrA-tagged substrates) do not require this domain for direct recognition [50, 52, 69]. For example, λ O (a replication protein of bacteriophage λ) carries an N-terminal degradation motif (N-motif 1, NH₂-TNTAKI), which is specifically recognised by the N-terminal domain of ClpX [52, 96, 99]. Indeed deletion of this domain (from ClpX) inhibits the ClpP-mediated degradation of λ O [52], which is proposed to result from the low affinity of this class of substrate to the axial loops on ClpX. Tethering of this class of substrate, by the N-terminal domain, is likely to increase the effective concentration of the substrate, near the pore of ClpX. As a result, despite their low affinity to the pore loops, high affinity to the N-terminal domain promotes their engagement by the pore and, consequently, their efficient degradation. The N-terminal domain is also involved in the recognition of the adaptor proteins, SspB and UmuD, and substrate proteins such as MuA (C-motif 2, Fig. 1.7), which appear to share a conserved motif [50, 52, 54]. Importantly however, the adaptor proteins are not degraded by ClpXP, presumably because they are not recognised by the pore-1 motif of ClpX.

Other Degradation Tags

Currently, the substrate recognition motifs for ClpA are only poorly defined. The first ClpAP substrate to be identified was ClpA itself [100]. Interestingly, although the recognition motif within ClpA was originally proposed to be located at the

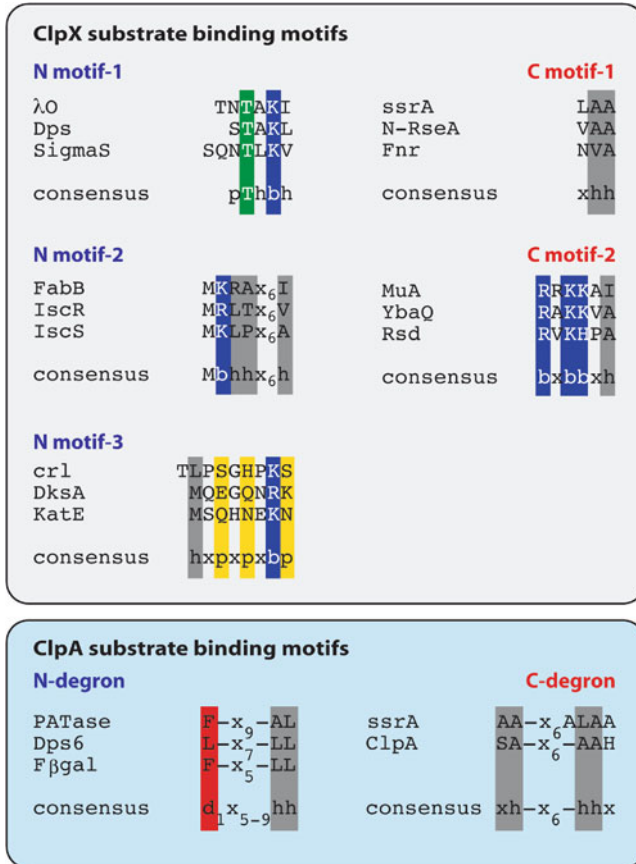


Fig. 1.7 Substrate-binding motifs for ClpX and ClpA. In general, AAA+ proteases recognise either the N- or C-terminus of a substrate, as such several motifs have been defined for both ClpX and ClpA. ClpX Substrate recognition by can be divided into five broad groups, three N-terminal motifs (*N motif-1, -2 and -3*) and two C-terminal motifs (*C motif-1 and -2*). In contrast only two ClpA recognition motifs have been observed for ClpA (*N-degron and C-degron*)

N-terminus of ClpA, it was later shown to be C-terminal [101]. Interestingly, this motif within ClpA shares some similarity with the, well characterised, model degron – the SsrA tag (Fig. 1.7). ClpA has also been shown to recognise proteins via an N-terminal recognition motif but not an internal motif [102, 103]. The N-terminal recognition motifs can be classified into two groups, those that require the adaptor protein (ClpS) – N-end rule substrates, and those that do not. Currently, only a single substrate containing an N-terminal recognition sequence has been identified [104], and consequently a motif has not been defined. In contrast, several N-end rule substrates, both natural and model substrates have been identified and hence a motif for ClpA binding of these substrates has been proposed [74, 78].

The ClpA recognition motif within N-end rule substrates is a dihydrophobic element, located between five and nine residues from the primary destabilising residue at the N-terminus of the protein [74, 105]. Interestingly, one of the N-end rule substrates, Dps (DNA protection during starvation), which protects DNA from reactive oxygen species, contains two N-terminal recognition motifs. One motif is created after endoproteolytic cleavage of the first five residues of Dps, to generate Dps₆₋₁₆₇ and is required for recognition by ClpS and ClpA [78, 79], the other N-terminal motif is created following cleavage of the N-terminal Met by methionine aminopeptidase (MetAP), to generate Dps₂₋₁₆₇ which contains a ClpX (Nmotif-1) within the first five residues of Dps [96].

Substrate Recognition by AAA+ Proteases (Direct Recognition Versus Indirect or Adaptor Mediated Recognition)

Although the recognition of most protein substrates occurs by direct interaction with the unfoldase, some protein substrates require additional recognition factors to direct them to the protease for degradation. In the following sections, we will describe the molecular details of substrate recognition by the unfoldase and/or delivery by adaptor proteins, using a number of well-characterised examples.

Direct Recognition by ClpX (e.g. Recognition of SsrA Tagged Proteins)

In *E. coli*, the SsrA tag is composed of 11 amino acids (AANDENYALAA), however recognition of this tag by ClpX, only requires the last two alanines and the C-terminal α -carboxylate [106]. In contrast to some ClpX substrates (e.g. λ O), recognition of the SsrA-tag by ClpX, does not involve the N-terminal domain. Consistent with the idea, removal of the N-domain of ClpX, did not alter the ClpP-mediated degradation of SsrA-tagged proteins [50, 52]. Rather, the SsrA-tag is specifically recognised by loops in, or near to, the axial pore of the AAA+ module. Indeed, three sets of pore loops in ClpX (RKH, pore-1 and pore-2, see Fig. 1.5) have been implicated in binding the SsrA tag [71, 107]. The RKH loops, as the name suggests, contains the tripeptide motif (RKH), which surrounds the entrance to the ClpX pore. The positively charged RKH loops are proposed to attract negatively charged sequences (i.e. the charged C-terminal α -carboxylate of the SsrA-tag) to the pore of ClpX [99]. Accordingly, mutations that reduce the positive charge of the RKH loop, reduced binding to SsrA-tagged proteins (or substrates containing a C motif-1), whilst simultaneously improved the binding of substrates containing a positively charged motif [99]. The pore-1 and pore-2 loops, in contrast to the RKH loop, interact with the two last alanine residues of the ssrA-tag [107, 108]. The pore-1 loop of ClpX

contains the highly conserved GYVG motif, which plays a central role in substrate translocation across the pore and into the degradation chamber [59–61, 109]. Based on a number of mutations and series of crosslinking experiments, the pore-2 loops were shown to specifically interact with the terminal alanines of the SsrA-tag [108]. Interestingly, neither the RKH nor the pore-2 loops are conserved in human mitochondrial ClpX [108]. As such, human ClpX is unable to recognise proteins tagged with the *E. coli* SsrA tag. However, a crucial role for these loops in the recognition of SsrA-tagged was elegantly demonstrated by Sauer and colleagues by grafting the *E. coli* ClpX RKH and pore-2 loops onto human ClpX creating a chimeric ClpX protein [108]. Strikingly, when both the RKH loops and pore-2 loops from *E. coli* ClpX were grafted onto human ClpX, the resulting chimeric proteins was able, not only to recognize the SsrA-tagged substrates but also to deliver them to ClpP for degradation [108]. Interestingly, grafting of only the RKH or pore-2 loop, was insufficient to promote recognition of the SsrA-tag. Collectively, these results demonstrated the importance of both pore loops in the recognition of SsrA-tagged substrates.

Indirect Recognition (Adaptor Mediated Recognition)

As mentioned above, the recognition of some protein substrates by the unfoldase, either requires or is modulated by an additional component – known as an adaptor protein. In general, an adaptor protein acts as a bridge between the substrate and the unfoldase. As such, adaptor proteins invariably exhibit two separate activities; (i) substrate recognition and (ii) unfoldase docking, however in some cases the adaptor protein is also proposed to activate either the substrate or the unfoldase for delivery to the protease for degradation [42, 110, 111]. Typically, the adaptor protein is released and recycled in this process without being degraded, although in some cases the adaptor protein (e.g. MecA) is also degraded by the protease complex (i.e. ClpCP), which acts a negative feedback loop to control the turnover of the substrates delivered by this adaptor protein. To date, four adaptor proteins have been identified in *E. coli*, three of which (SspB, UmuD and RssB) deliver specific protein substrates to ClpXP [54, 112, 113] while a single adaptor protein (ClpS) is required for the delivery of a specific class of substrates to ClpAP [40, 114]. SspB increases the affinity of ClpX to SsrA tagged proteins [112]. RssB is essential in bacteria for ClpXP-mediated degradation of the stationary phase sigma factor, σ^S [113, 115, 116]. Interestingly, four adaptor proteins have also been identified in *B. subtilis*. However, in contrast to the adaptor proteins from *E. coli*, the vast majority of *B. subtilis* adaptor proteins (MecA, McsB and YpbH) function together with ClpC [45, 117–121], and only a single adaptor protein (YjbH) has been identified to function with ClpX [122, 123]. Surprisingly, with the exception of MecA and YpbH, the remaining adaptor proteins share little, to no, sequence homology and hence each adaptor protein is likely to function via a unique mechanism.

SspB (A Multi-functional Adaptor Protein)

SspB is certainly the best characterised ClpX adaptor protein and arguably the best characterised bacterial adaptor protein to be studied. It was first identified, in *E. coli* as a ribosome-interacting protein that specifically modulates the ClpXP-mediated turnover of SsrA-tagged proteins [112]. Subsequently, SspB was also shown to recognise and deliver another ClpX substrate (i.e. RseA) for ClpP-mediated degradation [76] for a recent review see [6]. Although the distribution of SspB homologs is largely limited to γ - and β -proteobacteria an ortholog of SspB, termed SspB α has also been identified *Caulobacter crescentus* and other α -proteobacteria [124, 125]. Interestingly, despite the poor sequence homology, the overall fold of SspB α is similar to *E. coli* SspB [124]. Nevertheless, in contrast to *E. coli* SspB, *C. crescentus* SspB α appears to be optimised for binding to the SsrA-tag. In the case of *E. coli* SspB, the protein is composed of two functional regions separated by a long unstructured segment (~40–50 residues long). The N-terminal region of SspB (~110–120 residues long) forms a dimeric module, which is involved in binding of the SsrA-tag [50, 126, 127]. This substrate-binding domain is tethered to ClpX, via a short motif (termed the ClpX-binding region (XBR)), located at the C-terminus of SspB [50, 127]. The XBR of SspB forms an anti-parallel β -sheet with the N-terminal ZBD of ClpX [53]. Indeed, it has been proposed that both XBRs (from the SspB dimer) interact simultaneously with two ZBDs on ClpX – a mode of attachment that places the SspB-bound cargo in an ideal position for interacting with the pore residues in the ClpX hexamer. Hence, SspB tethers the substrate to ClpX thereby increasing the local concentration of SsrA-tagged substrates near the ClpX pore [50, 53, 127–129] (Fig. 1.8). Importantly, both the unfoldase and the adaptor protein recognise exclusive regions within the SsrA tag (AANDENYALAA). The unfoldase recognises the AA motif (C motif-1) at the C-terminus of the SsrA tag (Fig. 1.7), while SspB binds towards the N-terminal end of the SsrA-tag (AANDxxY). In contrast, the ClpA binding motif within the SsrA-tag (AAxxxxxALA) overlaps with the SspB binding and as such SspB inhibits the ClpAP-mediated degradation of SsrA-tagged substrates [50, 130]. As a consequence, SspB-mediated tethering of the SsrA-tag to ClpX results in an increased affinity of the substrate for ClpX, and hence an improved rate of degradation [129]. As such, SspB is likely to play an important role in the delivery of substrates present at low concentrations as tethering to ClpX, effectively increases the local concentration of the substrate. Consistent with this substrate-tethering model, mutation of the ClpX recognition motif within the SsrA-tag (i.e. replacement of LAA with DAS, termed the DAS-tag), significantly reduce the ClpXP-mediated degradation of substrates bearing this tag, while, the addition of SspB improved the recognition and degradation of substrates bearing this modified DAS-tag by more than 100-fold [131].

RssB

In contrast to SspB, RssB is a dedicated adaptor protein that is uniquely responsible for the recognition of a single substrate – the general stress transcription factor SigmaS (σ^S , also referred to as RpoS). RssB (also known as SprE (stationary phase regulator)

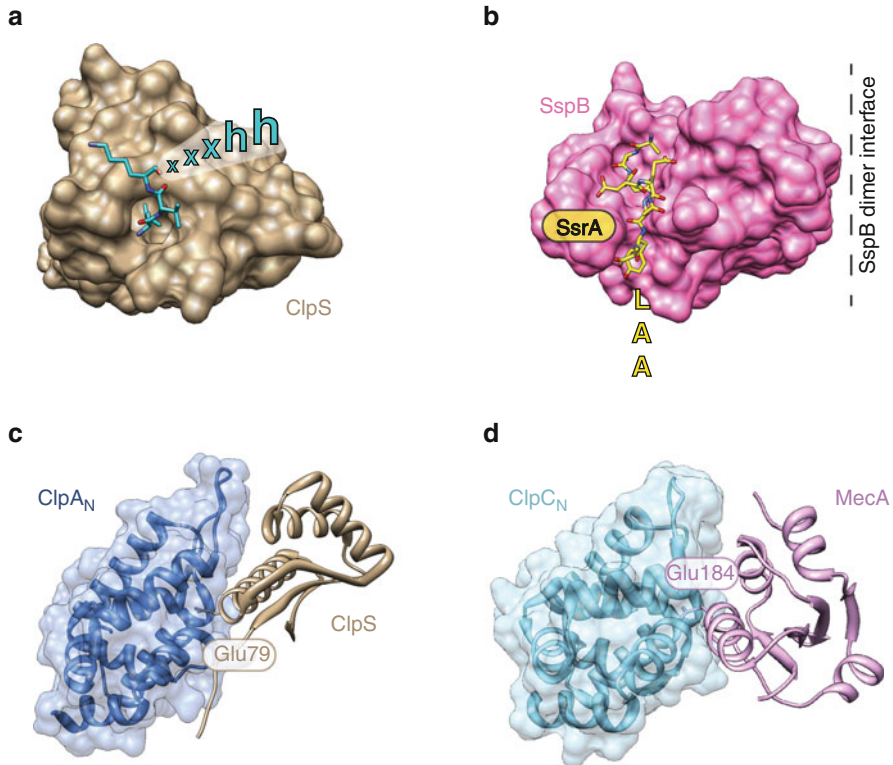


Fig. 1.8 Adaptor proteins. (a) ClpS (*tan*) contains a small substrate-binding pocket for the recognition of proteins bearing a primary destabilising residue at their N-terminus. This binding pocket exhibits exquisite specificity and not only forms a number of critical H-bonds with the α -amino group of the N-terminal residue of the substrate (*blue*), but also forms hydrophobic interactions with the side-chain of the N-terminal residue. The substrate extends away from ClpS and reaches towards the unfoldase, binding to ClpA is proposed to occur through the dihydrophobic (hh) element. (b) SspB (*pink*) forms a more permissuuous peptide-binding groove, which can accommodate peptides in different orientations. In the case of SsrA-tagged proteins, the substrate extends away from SspB and towards the pore loops of ClpX, which interact with the LAA motif. (c–d) Both ClpS (*tan*) and MecA (*pink*) interact with the N-terminal domain of ClpA (*blue*) and ClpC (*light blue*), respectively. An α -helix within the adaptor protein, contains a critical Glu residue which projects into the conserved pocket within the appropriate N-domain

in *E. coli* or MviA in *Salmonella typhimurium*) was first identified in *E. coli* using a genetic screen to discover genes involved in the RpoS expression and/or activity [115, 116]. RssB is a member of the two-component response regulator family and was the first family member to be shown to play a role in protein turnover. As a member of the response regulator family, RssB is phosphorylated on a highly conserved aspartate residue (D58). Phosphorylation of RssB at D58, stimulates binding of σ^S , resulting in the formation of a stable 1:1 complex [132]. Mutations that inhibit

phosphorylation of RssB result in reduced binding to σ^S , and hence an increased stability of σ^S , both *in vitro* and *in vivo*. In contrast to SspB, which merely enhances the kinetics of substrate recognition, RssB is essential for the recognition of σ^S by ClpX [113]. Indeed it has been proposed that binding of RssB to σ^S triggers a conformational change in σ^S , which exposes a previously concealed ClpX recognition motif, however the mechanistic details of such a model are yet to be confirmed. For further details on the proteolytic control of the general stress response in bacteria refer to [7].

UmuD

The third and final, ClpX-specific adaptor protein in *E. coli*, is UmuD. In response to DNA damage, the first 24 residues of UmuD are auto-catalytically cleaved, in a RecA-dependent fashion. Following cleavage, the resulting protein (termed UmuD'), can form both homo- and hetero-oligomers [133]. As a heterodimer, UmuD/UmuD' forms a component of the error-prone DNA polymerase V, which is able to bypass DNA lesions in the process of DNA replication and hence facilitates the cells recovery following DNA damage. Since this activity is necessary at times of DNA damage, but toxic under normal growth conditions, it is important that the cellular levels of UmuD' (and hence UmuD/D' oligomers) be carefully controlled during and after recovery. Indeed, this is elegantly achieved by the cell, as the N-terminal region of UmuD serves as a ClpX tethering sequence for delivery of UmuD' to ClpXP when present in an UmuD/D' complex [134]. Like the XBR of SspB, this region can bind to the ZBD of ClpX, but not as a degradation tag rather as a specific adaptor protein for the delivery of UmuD [54, 134].

Indirect Recognition by ClpA (Recognition of N-End Rule Substrates)

ClpS and the N-End Rule (A Specific ClpAP-Mediated Substrate)

In contrast to ClpX, which uses three different *E. coli* adaptor proteins, only a single adaptor protein (ClpS) has been identified for ClpA. Although ClpA appears to use only a single adaptor protein, this adaptor protein exhibits broad activity over its cognate unfoldase. Indeed, ClpS is able to regulate ClpA substrate selection, both negatively and positively [40, 114]. Originally identified as an inhibitor of ClpA auto-degradation, both *in vitro* and *in vivo*, and a negative regulator of ClpAP-mediated degradation of substrates bearing an SsrA-tag [40], ClpS was also shown to be an essential component of the N-end rule pathway [114].

The N-end rule pathway, originally identified in *Saccharomyces cerevisiae*, by Alexander Varshavsky's lab, is a highly conserved protein degradation pathway that is responsible for the recognition and degradation of proteins bearing a specific

“destabilising” residue at the N-terminus [135, 136]. This pathway determines the half-life of a protein based on the N-terminal residue of that protein, which may be classified as “stabilising” or “destabilising”. To date, this pathway has been identified in bacteria, plants and mammals and although the details of the various pathways differ, from one organism to the next, each pathway shares a number of common principals [135, 137–139]. In *E. coli*, like other organisms, the pathway is hierarchical, and destabilising residues can be separated into two classes (primary and secondary) [74]. Primary destabilising residues (L, F, W and Y) are recognised directly by the bacterial N-recognin, ClpS [114], while secondary destabilising residues (R, K and M) must first be converted to primary destabilising residues by the enzyme Leu/Phe-tRNA-protein transfersase (LFTR) before they are recognised by the adaptor protein [78, 140]. Interestingly, the first clue for a role of ClpS in the N-end rule pathway came from the structure of ClpS and comparison to the secondary structure of the human N-end rule recognition component (N-recognin), the E3-ligase, UBR1 [42, 141]. From this bioinformatic analysis, despite very low sequence homology, Lupas and colleagues proposed that ClpS was involved in the N-end rule pathway in bacteria [141]. Consistently, the crystal structure of ClpS (in complex with the N domain of ClpA) identified two conserved regions, one for interaction with the N-domain of ClpA and the other proposed to be involved in a substrate interaction [41, 42]. Subsequent biochemical and structural analysis confirmed that ClpS was indeed essential for the recognition of N-end rule substrates and that the second conserved region within ClpS was the N-degron binding site [142–144].

ClpS, like most characterised adaptor proteins is a small protein composed of two regions. The C-terminal domain of ClpS is the “workhorse” of the protein, it is responsible, not only for recognition of the substrate but also for docking to the N-terminal domain of ClpA [40–42, 114]. Despite both of these functions being located on the C-terminal domain of ClpS, this domain alone is neither sufficient for the inhibition of substrates bearing an SsrA-tag nor the delivery of N-end rule substrates [41, 110], suggesting that the N-terminal region of ClpS plays a crucial role in activation of ClpA. Hence in contrast to SspB, which merely modulates the affinity of ClpX for recognition of the SsrA-tag, the adaptor protein ClpS alters the substrate specificity of ClpA, by activating the unfoldase for recognition of N-degron bearing substrates. In summary, a substrate bearing an N-terminal primary destabilising residue, is bound by a small hydrophobic pocket on the surface of ClpS (Fig. 1.8). Importantly, this pocket exhibits exquisite specificity – it forms a number of important hydrogen bonds with both the α -amino group of the N-terminal residue and the carbonyl oxygen of the peptide bond, as well as several hydrophobic interactions with the side chain of the N-terminal amino acid [142–144]. Following recognition of the substrate by the adaptor protein, the substrate-ClpS complex docks to the N-terminal domain of ClpA [40–42]. Next, the N-terminal region is proposed to activate, an as yet undefined region of ClpA, for recognition of the N-degron bearing substrate [110]. The unfoldase (ClpA), then recognises a hydrophobic region in the substrate approximately ~5–9 residues downstream of the primary destabilising residue (Fig. 1.7) [74, 78, 105].

MecA

Of the three known ClpC-adaptor proteins, *MecA* is currently the best characterised. It was first discovered in a genetic screen for repressors of competence development (which is a physiological state that permits *B. subtilis* cells to actively import DNA). In non-competent cells, the “competence” transcription factor (ComK) is recognised by *MecA* and targeted for degradation by ClpCP. Competence is triggered by the accumulation of a small peptide (ComS), which binds to *MecA* and thereby inhibits the *MecA*-dependent degradation of ComK by ClpCP [145]. Interestingly, *MecA* is not only involved in the development of competence through the regulated degradation of ComK but has also been proposed to be involved in general protein quality control, through the ClpCP-mediated degradation of misfolded and aggregated proteins [120]. Similar to most other adaptor proteins, *MecA* is composed of two regions: an N-terminal domain, which is responsible for substrate recognition (i.e. ComK and ComS) and a C-terminal domain, which is required for docking to the unfoldase [146]. Interestingly, in contrast to other characterised adaptor proteins, docking of *MecA* (and hence substrate delivery to the protease) requires both the N-domain and the M-domain of ClpC [36, 48]. Despite the additional requirement for *MecA* binding to ClpC (i.e. to the M-domain), the mode of docking of *MecA* to the N-domain is strikingly similar to that of ClpS with the N-domain of ClpA [41, 42]. Indeed both adaptor proteins (ClpS and *MecA*) use a single α -helix to interact with the same region of the N-domain, and stabilise the complex by the formation of several H-bonds (Fig. 1.8). Interestingly, *MecA* is absent in cyanobacteria and ClpC was shown to cooperate with the adaptor protein ClpS [147]. Consistently, the distribution of *MecA* and ClpS appears to be mutually exclusive throughout evolution.

Substrate Processing by AAA+ Proteins

Substrate translocation is a basic mechanical process of all AAA+ proteins. This process is performed solely by the AAA+ module of the unfoldase, and like substrate binding has been extensively studied using both ClpA and ClpX as a model AAA+ protein. In recent years however, there have been many advances in this area of research by several researchers, including numerous contributions by the laboratory of Robert Sauer and Tania Baker to study the basic mechanism of action of ClpX.

Substrate Unfolding and Translocation

In order for a folded protein to enter the degradation chamber of ClpP, it must first be unfolded by the ATPase component. Although the pore of ClpX is large enough to simultaneously accommodate two or three peptide chains, most folded proteins are too large to enter [148]. As such, the narrow size of the ClpX pore prevents diffusion

of folded proteins through ClpX and hence prevents the uncontrolled degradation of folded proteins by the ClpXP protease. Therefore protein substrates must first be unfolded, to enter the proteolytic chamber of ClpP. To achieve this, the unfoldase component converts the energy released from the binding and hydrolysis of ATP into a pulling force. This pulling force is responsible for the global unfolding of the substrate by threading it through the unfoldase pore, and into the degradation chamber of ClpP in a vectorial manner [95, 149].

The pulling force, generated by nucleotide-driven changes in the structure of ClpX is proposed to be transmitted to the substrate via movement of the pore-1 loops (Fig. 1.7). Indeed, the location of each pore-1 loop, within the central pore of the ClpX hexamer was shown to vary depending on the nucleotide bound state of the subunit [55]. As such, it has been proposed that ATP binding and hydrolysis drives conformational changes in the unfoldase, which result in movement of the pore-1 loops up and down the central pore [107, 150]. As these loops (in particular the highly conserved tyrosine residue) interact with the substrate's polypeptide chain, their movement along the pore provides the pulling force that is necessary for substrate unfolding and translocation. Do the subunits in a ClpX hexamer have to operate in a concerted fashion to promote successful unfolding and translocation of protein substrates? To examine this question Sauer and colleagues employed a method first used by the lab of Art Horwich, to study the role of individual subunits in GroEL [151]. In this case however, Sauer and colleagues created a single-chain hexamer of ClpX (lacking the N-terminal ZBD) by fusing six copies of *clpX* (lacking the sequence coding for the N-terminal ZBD) into a single gene [152]. This elegant experimental setup allowed the incorporation of specific mutations into various different ClpX subunits within the hexamer. Specifically, mutations in either the Walker B motif (E185Q) – which prevents ATP hydrolysis, or the sensor-2 motif (R370K) – which prevents both ATP hydrolysis and uncouples conformational changes linked to ClpP- and substrate-binding, were combined with wild type subunits and the degradation of SsrA-tagged substrates was examined [152]. Consequently, ClpX hexamers were created which contained either a single wild type subunit, or two wild type subunits and so on. Remarkably, the degradation rate increased linearly with the amount of the wild type subunits in a hexamer. For example, a ClpX hexamer that contained one wild type subunit led to degradation of an SsrA-tagged substrate 17% as fast as a wild type hexamer. In accordance, two wild type subunits in a hexamer resulted in a degradation rate that was 30% of that observed for a single-chain hexamer that was constructed of six wild type subunits. Moreover, in all cases, degradation was performed at a similar energetic cost (i.e. the amount of ATP hydrolyzed per substrate). These results indicated that even a single active subunit in a hexamer can promote efficient unfolding and translocation of substrates by ClpX and that concerted or sequential activity of multiple subunits is not essential for degradation.

Degradation of folded substrates proceeds much slower than degradation of unfolded substrates, suggesting that substrate unfolding is a rate-limiting step for proteolysis by AAA+ proteases. This principle was elegantly demonstrated using the I27 domain of human titin as a substrate [92]. Titin-I27 was converted into a

ClpX substrate by creating a genetic fusion of titin-I27 with SsrA. The I27 domain of titin is extremely resistant to mechanical unfolding [153] and consistent with this, its degradation by ClpXP is relatively slow [92, 94]. Remarkably, an unfolded variant of titin-I27-SsrA can be obtained, simply by carboxymethylation of its two cysteine residues [92]. This simple chemical modification completely unfolds titin-I27 without altering its solubility. This permitted a direct comparison of the degradation kinetics of the substrate with respect to its folded state (i.e. either stably folded or unfolded). Interestingly, both the folded and unfolded substrates had a K_M of $\sim 1 \mu\text{M}$, similar to that observed for other SsrA tagged proteins. By contrast, the energetic cost for degradation of different substrates by ClpXP varied dramatically. For instance, the degradation of native titin-I27 required ~ 600 molecules of ATP, while the degradation of an unfolded mutant of titin-I27 only required ~ 100 molecules of ATP, suggesting that the cost of titin-I27 unfolding is ~ 500 ATP. Interestingly however, the rates of degradation do not correlate with the global thermodynamic stability of a substrate but rather seem to depend on the local stability of the region to which the recognition tag is attached [94, 154, 155]. In summary, the current model suggests that following binding, ClpX pulls on the degradation tag in an attempt to unfold the substrate. In some cases unfolding of the substrate may require multiple rounds of ATP hydrolysis until a power stroke of ClpX coincides with transient unfolding of a structural element near the degradation tag. When this occurs, ClpX can initiate substrate translocation and complete substrate unfolding very rapidly and with a high degree of cooperativity. As mentioned previously substrates may be recognised from either an internal site or from the N- or C-terminus of the protein [46]. Not surprisingly, substrate translocation may occur in either direction (i.e. from N-terminus to C-terminus or visa versa). Strikingly, single molecule experiments indicate that substrate unfolding eventually results from a single ClpX power stroke [154]. Following the initial unfolding event, substrate translocation proceeds rapidly and without considerable specificity [156]. Indeed, ClpX was shown to efficiently translocate a variety of different polymers, including homopolymeric tracts of glycine, proline and lysine non-amino-acid aliphatic chains. In addition, ClpX can carry out translocation of a polypeptide from the N-terminus to the C-terminus as efficiently as in the opposite direction.

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