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Regulated Proteolysis in Bacteria

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Abstract

Regulated proteolysis is a vital process that affects all living things. Bacteria use energy-dependent AAA+ proteases to power degradation of misfolded and native regulatory proteins. Given that proteolysis is an irreversible event, specificity and selectivity in degrading substrates are key. Specificity is often augmented through the use of adaptors that modify the inherent specificity of the proteolytic machinery. Regulated protein degradation is intricately linked to quality control, cell-cycle progression, and physiological transitions. In this review, we highlight recent work that has shed light on our understanding of regulated proteolysis in bacteria. We discuss the role AAA+ proteases play during balanced growth as well as how these proteases are deployed during changes in growth. We present examples of how protease selectivity can be controlled in increasingly complex ways. Finally, we describe how coupling a core recognition determinant to one or more modifying agents is a general theme for regulated protein degradation.

Contents

INTRODUCTION

Regulated protein degradation is a vital process that affects all biological pathways. Because proteolysis is an irreversible event, the cell must take great care to avoid degrading proteins indiscriminately. As a consequence, energy-dependent proteases are finely tuned cellular machines that recognize substrates with exquisite sensitivity and selectivity.

In eukaryotes, proteins targeted for degradation are modified by the covalent linkage of ubiquitin, a small protein that is appended to a lysine residue on the target protein (see Reference 1 for a review). Following additional extension by the ubiquitin ligase families, the proteasome recognizes and degrades the target protein. In bacteria, regulated proteolysis is carried out by energy-dependent AAA+ (ATPases associated with cellular activities) proteases that use the power of ATP hydrolysis to recognize, unfold, translocate, and degrade substrates. Several energy-dependent proteases exist in bacteria: Lon, ClpXP, ClpAP, ClpCP, ClpEP, HslUV, and FtsH (2–4). The importance of these AAA+ proteases is highlighted by the defects in viability and virulence of bacteria deficient in one or more proteases (5, 6). For instance, bacteria lacking Lon are known to be filamentous and more sensitive to ultraviolet radiation than their wild-type counterparts (7). In the alpha-proteobacterium *Caulobacter crescentus*, ClpXP is essential, as mutants lacking this protease are arrested during the cell cycle (8). In the human pathogen *Vibrio cholerae*, Lon mutants were unable to compete with wild-type *V. cholerae* in colonizing the infant mouse intestine (9).

AAA+ proteases are composed of ATPase and peptidase domains with similar general architectures (10). In the case of Lon and FtsH, the two domains are encoded on a single polypeptide chain (11, 12). However, the Clp family of proteases encodes distinct hexameric ATPases (either ClpA or ClpX in gram-negative bacteria and ClpC or ClpE in gram-positive bacteria), which associate with ClpP, a sequestered 14-subunit peptidase, to form ClpXP, ClpAP, ClpCP, or ClpEP (2, 13). ClpP alone has the ability to degrade small peptides, but to degrade larger, more stable substrates, ClpP must associate with an unfoldase that harvests the energy of ATP hydrolysis to power degradation (13, 14). This is separate from the properties of non-energy-dependent proteases and peptidases that serve important recycling roles in the cell (15). We focus primarily on the energy-dependent AAA+ proteases.

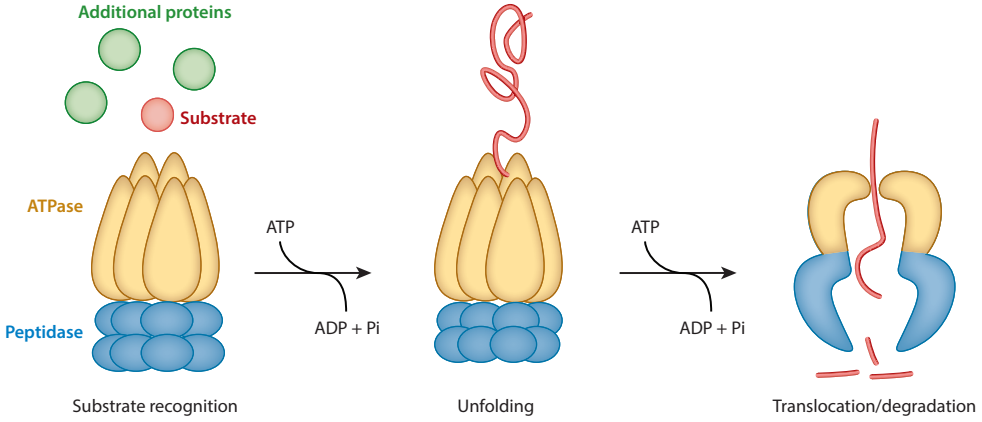


Figure 1

Energy-dependent proteases are composed of an ATP-hydrolysis active unfoldase domain and a chambered peptidase domain. Through successive rounds of ATP hydrolysis, a specific substrate protein is selected by the protease, unfolded by the ATPase domain, and translocated through a central pore to the peptidase chamber where it is degraded.

OPERATIONAL RULES OF PROTEOLYSIS

Regardless of architecture or function, bacterial AAA+ proteases seem to follow similar operational rules. In the most general case, regulated proteolysis requires recognition of an initial degradation determinant (also known as degrons), followed by complete degradation of the polypeptide in an ATP-dependent manner (**Figure 1**). The unfolding power and processivity of an AAA+ protease depends on both substrate and protease. For example, poorly folded substrates require less ATP hydrolysis for unfolding (16). As with many systems, there are exceptions, for example, slippery sequences within certain substrate proteins can stall proteases, resulting in release of partially processed species (17, 18). By appending the same substrate with different degrons, the unfolding and processivity of the known bacterial AAA+ protease classes were shown to vary more than two orders of magnitude (19), with Lon being the "worst" unfoldase and ClpAP being the "best." Because unfolding parameters can vary wildly depending on the specific fold of the substrate, we are cautious in generalizing these results to all substrates, but single-molecule experiments have recently shown similar correlations between some of these machines (20–22).

Due to the processive nature of these proteases, the most important governing feature in vivo is likely the pioneering round of substrate engagement, as once a substrate is committed for degradation it is unfolded and translocated relatively quickly (22). This initial commitment is a combination of the specificity of the protease for a given class of substrates and the ability of those substrates to be recruited to the protease. For any AAA+ protease to successfully degrade a substrate, there must be initial recognition of some determinant on the substrate for the protease to start pulling on. This intrinsic recognition can be modified through inhibition or activation by additional factors or the substrates themselves in complex ways that depend on the needs of the cell. These needs can include quality control, as damaged or misfolded proteins must be cleared before they elicit toxic effects. However, energy-dependent proteases are also playing a major role in maintaining protein homeostasis during balanced growth and during physiological transitions, such as stationary phase or sporulation. Although the mechanisms proteases employ for these distinct arms of degradation may differ, the general theme of linking a core recognition determinant to a modifier seems to be common.

PROTEASES AS QUALITY-CONTROL RESPONDERS

Bacteria live in a dynamic, constantly fluctuating environment where they are subject to proteotoxic stressors, such as heat or oxidative stress. Because stress conditions require a swift response, regulated proteolysis allows bacteria an effective way to get rid of damaged proteins rapidly, without having to wait for protein removal by dilution through cell division (14, 23).

In addition, many stresses lead to the accumulation of misfolded proteins, a problem that needs to be addressed by the cell before lethal consequences ensue. The response to stress can be thought of as a competition between rescuing factors, such as chaperones or repair enzymes, which seek to restore proteins, and proteases, which seek to degrade them (**Figure 2*a***). A central challenge for the cell therefore lies in determining when a protein is terminally damaged or misfolded and when rescue attempts should be abandoned for proteolysis (24).

Recognizing Failed Quality Products Through Specific Tags: The ClpXP Protease

Misfolded proteins are cleared by Lon in bacteria as well as in the mitochondrial matrix in eukaryotes (25, 26). In *Escherichia coli*, Lon is thought to be responsible for the degradation of approximately 50% of misfolded proteins (27), which suggests that the protease recognizes general motifs in misfolded proteins with little sequence specificity. By contrast, the bacterial ClpXP protease is far more selective and requires a specific degron sequence, such as the ssrA tag, to recognize a substrate (28, 29). These two enzymes exemplify different mechanisms used to ensure degradation of poor-quality proteins.

One of the best-studied examples in bacteria of regulated proteolysis is the recognition of the ssrA tag by ClpXP following *trans*-translation, a mechanism by which stalled ribosomes are rescued upon recruitment of tmRNA and nascent polypeptides are tagged with the ssrA peptide (30). Because these stalled ribosomes often arise from damaged messenger RNAs (mRNAs) that lack a stop codon, the resulting polypeptide products cannot be complete. Therefore, the presence of the ssrA tag is itself the signal for a poor-quality protein. Recognition of the ssrA tag by ClpXP is highly specific, with even a single amino acid substitution abolishing substrate recognition (28). The amount of ssrA-tagged proteins is staggering, with some estimates indicating that an ssrA tag is appended in approximately 1 in every 20 translation events (31). During starvation, ribosome stalling and mRNA cleavage are enhanced, resulting in further need for the *trans*-translation system (32). Activation of certain endogenous toxins, such as MazF and RelE, can induce rampant mRNA cleavage as well that is counteracted by tmRNA (33). In these cases, the need for clearance of ssrA-tagged proteins becomes even more urgent to eliminate the surge in truncated polypeptides.

The ClpXP protease is fully capable of degrading ssrA-tagged proteins. However, the adaptor protein SspB enhances the ability of ClpXP to recognize and degrade ssrA-tagged substrates (34). SspB forms a homodimer, with each subunit containing a substrate binding domain that binds ssrA-tagged proteins, and a disordered C-terminal tail that interacts with ClpX. Efficient delivery of ssrA-tagged substrates requires both tails on each subunit of SspB to interact with ClpX, which tethers substrates to the protease to increase effective substrate concentration (35). Thus, the ssrA tag is the fundamental protease recognition determinant with the SspB adaptor acting as a modifier of this recognition by serving as a passive tether (**Figure 2*b***).

In addition to enhancing the ability of ClpXP to degrade ssrA-tagged substrates, SspB also promotes degradation of N-RseA, the N-terminal fragment of the stress response protein RseA (13, 36). During normal conditions, RseA binds σ^E, preventing it from activating transcription. However, during the envelope-stress response, RseA is cleaved by proteases, freeing the N-terminal segment of RseA in complex with σ^E. SspB then delivers N-RseA to the ClpXP protease, leaving

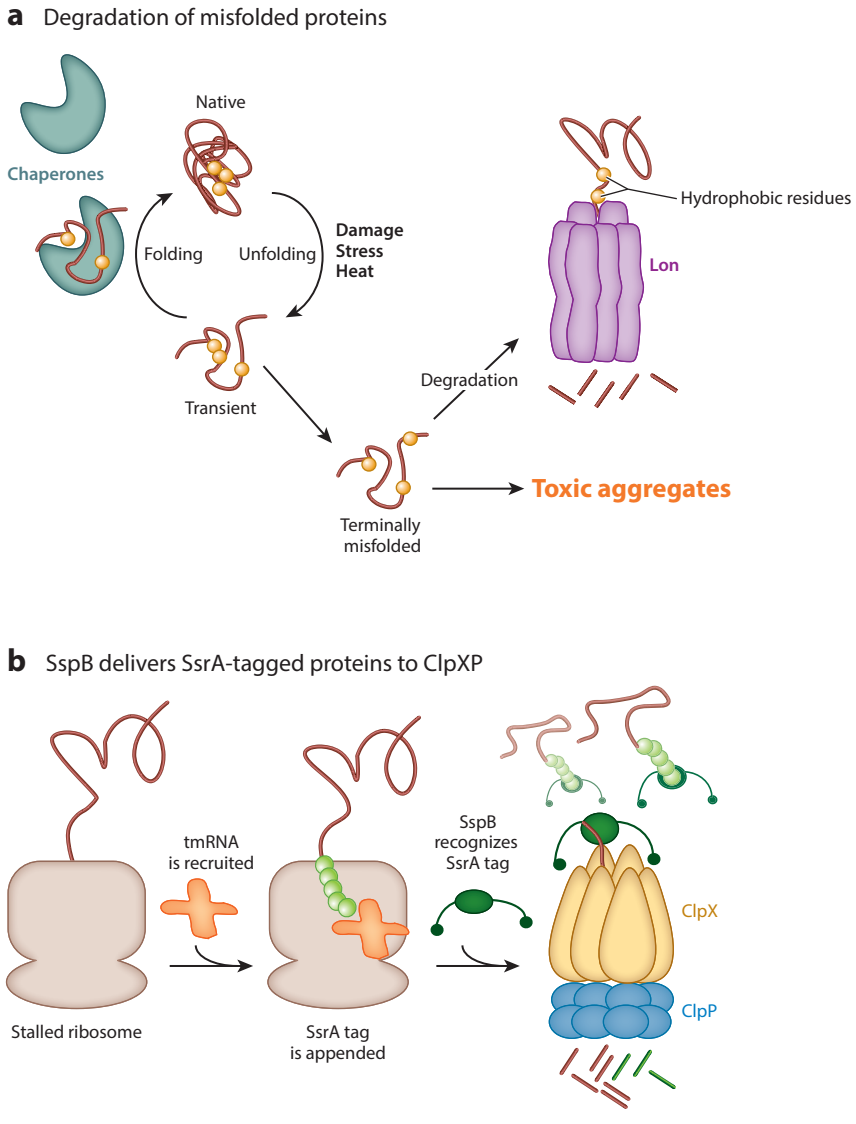


Figure 2

Proteases can survey protein quality in the cell. (*a*) Competition between chaperones and proteases dictates the fate of proteins. Proteases, such as Lon, must be able to distinguish between normal protein dynamics with transient excursions into non-native states and terminally misfolded proteins that must be degraded before forming toxic aggregates. Lon recognizes hydrophobic motifs (*yellow circles*) that are usually buried in the core of a native protein. These motifs are exposed more persistently for misfolded proteins than during the transient fluctuations of properly folded proteins, allowing Lon to recognize and degrade the terminally misfolded proteins. Chaperones contribute to this flux by binding misfolded proteins in an effort to refold them. (*b*) Following the process of *trans*-translation, in which an ssrA tag is appended to incomplete polypeptides, the adaptor SspB binds tagged substrates and tethers them to ClpXP, enhancing the protease's ability to degrade these substrates.

σ^E free to upregulate the envelope-stress response. Remarkably, there is no clear sequence similarity between the region of N-RseA that interacts with SspB and the region of the ssrA tag that binds SspB; indeed, binding of N-RseA is in the opposite orientation as that of ssrA (36). Having a single adaptor bind multiple substrates would enable a coordinated response across several pathways and, perhaps not surprisingly, other examples of adaptors enabling degradation of several substrates are now emerging (37–39). However, this multiplexing would eventually reach an upper limit as the need for selectivity begins to outweigh the advantages of coordinated degradation.

Recognizing Poor-Quality Proteins Without Specific Tagging: The Lon Protease

The cellular response to an acute stress must often occur at timescales faster than transcription and translation. Importantly, if the stress affects some of this central machinery, such as the fidelity of the ribosome, then the consequences of this stress must be repaired prior to restarting normal growth. Lon protease is uniquely suited to serve as a quality-control protease due to its ability to broadly recognize misfolded proteins and its ability to be allosterically activated.

Quality control through regulated proteolysis requires bacteria to discriminate between misfolded or damaged proteins and proteins that simply share features associated with compromised proteins, such as folding intermediates or during normal transient fluctuations in protein structures. Compared to other energy-dependent proteases, the Lon protease has the weakest unfolding capacity (19), but a surprisingly promiscuous substrate repertoire. Indeed, Lon seems to recognize hydrophobic residues on misfolded substrates that are typically buried in the native structure as its primary recognition determinant rather than any unique sequence motif (40; see also **Figure 2*a***and Reference 41 for a review).

To properly survey protein quality, Lon must be able to distinguish between fatally misfolded proteins and those that are in intermediate folding states. Given that terminally misfolded proteins are kinetically trapped, it seems likely that the lifetime of the exposed hydrophobic regions may be a key determinant for this type of quality-control surveillance. In this model, the shorter lifetime of exposed hydrophobic regions for a protein properly en route to the native state would set the lower limit of time that Lon would use to recognize a poor-quality protein. By extension, this means that the Lon-substrate complex would need to be sufficiently transient to be compatible with this discrimination; otherwise, Lon would erroneously destroy proteins in the process of being properly folded or those proteins that are fluctuating with normal dynamics. Thus, by combining a low-efficiency unfolding capacity with a broad recognition spectrum, the Lon protease gains selectivity in recognizing truly misfolded proteins. A similar model is thought to hold for eukaryotic quality control, where only persistent Hsp70 chaperone binding to a folding client recruits the C-terminal Hsp70-interacting protein (CHIP) ubiquitin ligase to target the client for ubiquitylation and degradation (42).

The ability of cells to use proteases and chaperones to ensure protein quality can also apply to the quaternary structure of complex assemblies. For example, individual subunits of protein complexes must be assembled in the correct stoichiometry to ensure function. Overflow of these subunits could be toxic, but AAA+ proteases are well-suited to destroy these unincorporated subunits. For example, degradation of the CcdA antitoxin by the Lon protease is inhibited when it is incorporated into the CcdAB complex (43) and degradation of subunits are often suppressed when complexes are fully assembled. In this respect, Lon ensures that active protein complexes maintain the proper stoichiometry.

Similarly, many protease substrates are DNA binding factors, including transcription factors, replication regulators, and components of polymerases (44–47). For some substrates, degradation

is only evident when the substrate is not binding DNA, suggesting again that a surveillance of the proper active complex (in this case the degree of DNA-bound species) affords the cell the ability to ensure destruction of proteins when they are not performing their function. We caution that interpretation of this phenomenon is more complex, as in some cases DNA can stimulate substrate recognition by the protease (46), and in the case of Lon, DNA can affect the protease directly (48, 49; see 47 for a review).

The allosteric activation of Lon is an intriguing aspect to consider in light of its role in quality control in the cell and its fundamental broad specificity. Many enzymes exhibit substrate-activity relationships in line with the classic Michaelis-Menten equation. The ClpXP and ClpAP proteases fall into this class, where increasing substrate initially results in linear increases of degradation rates until the enzyme becomes saturated and maximum reaction velocity (V_{max}) is achieved (**Figure 3*a***). In such cases, an underlying assumption is that the enzyme-specific activity is unchanged as substrate is added.

By contrast, the Lon protease has long been known to respond to substrate concentration in a cooperative manner (50), with the working model that substrates allosterically activate the Lon protease and increase its proteolytic activity. Intriguingly it has been shown that substrates not only activate Lon for their own degradation, but can also serve to activate Lon for degradation of other substrates (51). Thus, the behavior of Lon in the presence of two substrates could lead to regulation conducive to quality control. For example, suppose substrate 1 can only activate Lon at a higher substrate concentration, whereas substrate 2 has a higher affinity for Lon such that low concentrations of this substrate are needed to allosterically activate Lon (**Figure 3*b,c***). Titration of these substrates would show that at the same concentrations, substrate 1 is degraded more slowly than substrate 2. However, addition of substrate 2, even at concentrations lower than substrate 1, would cause shifting of the Lon population to the more active species, resulting in more rapid degradation of substrate 1 (**Figure 3*d***). Therefore, higher affinity substrates can effectively act as activators of lower affinity substrate degradation. In these cases, the basic recognition of the protease is modified by the presence of other substrates.

An intriguing extension of this biochemical framework is that it would result in the greatest activation of the Lon protease when demand is greatest in vivo. For example, during acute proteotoxic stress, the rapid increase in misfolded proteins would result in allosteric activation of Lon to eliminate these misfolded proteins, but also to degrade fully active regulatory proteins as part of the stress response. Such a case was recently reported in *Caulobacter*, with the Lon-dependent degradation of DnaA serving to halt cell-cycle progression during proteotoxic stress (52). This type of regulation would also make sense for a protease such as Lon given its ability to recognize features found in all proteins (such as hydrophobic residues), as persistently high activation of Lon would inevitably result in the destruction of proteins not needed for quality control. Indeed, overproduction of Lon in *E. coli* results in cell death, in part because of rampant degradation of antitoxin proteins (53). Finally, because Lon can be allosterically regulated by nonprotein molecules such as DNA, one can speculate that a surge in Lon activity upon allosteric activation could also be deployed under additional stress responses, such as during genotoxic stress where extended exposure of single-stranded DNA is a unique flag for DNA damage.

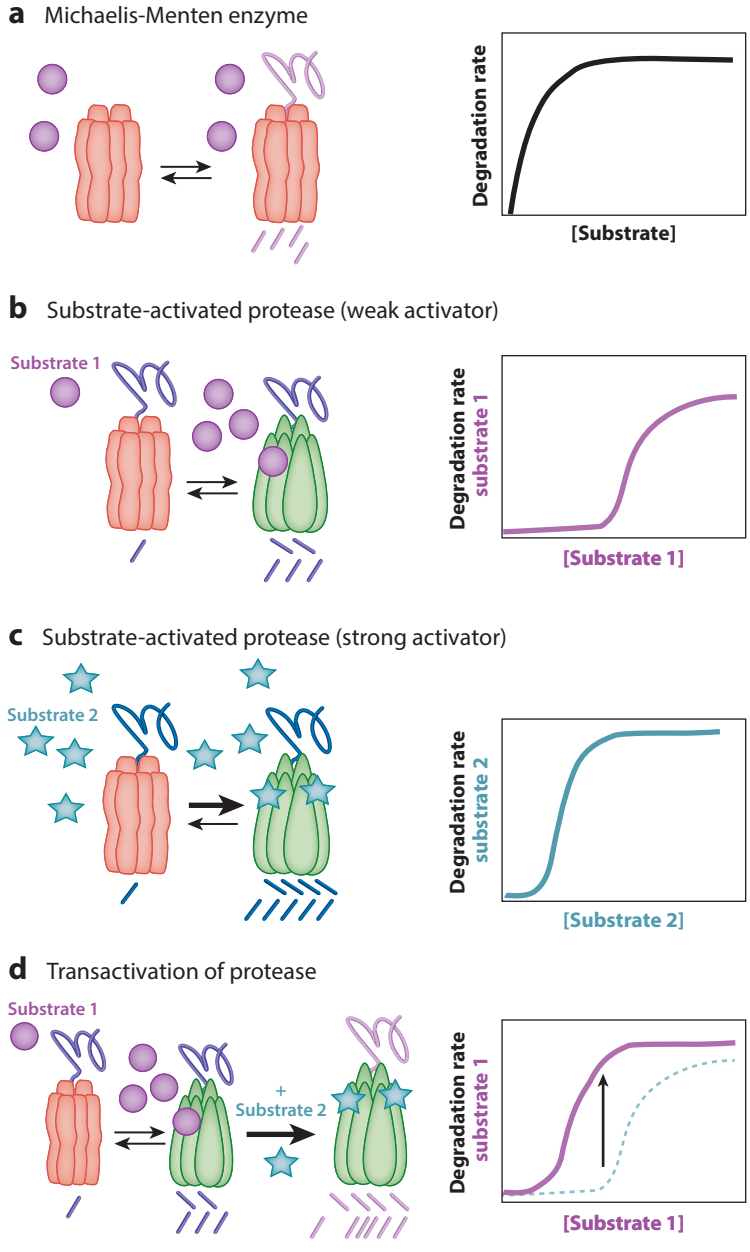
REGULATED PROTEOLYSIS DURING BALANCED GROWTH

Coordinating Proteolysis With Cell Division and Replication

The cell cycle involves a highly regulated sequence of events in which DNA is faithfully replicated and divided into daughter cells. Progression through the cell cycle requires regulated proteolysis

to ensure the timely degradation of regulatory proteins necessary to drive this irreversible process. In eukaryotes, the concerted activity of APC/C and SCF ubiquitin ligases enforce the selective tagging and ultimate degradation of many regulatory factors (54).

In *Caulobacter crescentus*, asymmetric cell division yields a motile, flagellated swarmer cell and a sessile stalked cell (23, 55). The stalked cell is replication-competent and can immediately commence DNA replication and enter the cell cycle. However, the swarmer cell must first shed its



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Lon is subject to allosteric regulation. (*a*) Many proteases, such as ClpXP and ClpAP, adhere to typical Michaelis-Menten kinetics, and adding increasing amounts of substrate will increase the rates of degradation until the protease becomes saturated and V_{max} is reached, resulting in the classic hyperbolic curve. This contrasts with Lon, which exhibits positive cooperativity upon increasing substrate concentration. The working model is that Lon exists in low (*red protease*) and high (*green protease*) activity states with substrate binding promoting the highly active state. (*b*) In the case of substrate 1, which binds Lon poorly, activation requires much higher concentrations of the substrate to shift Lon to the active state. (*c*) Substrate 2 has a strong affinity for Lon, and relatively low amounts of substrate are needed for activation. (*d*) If the concentration of substrate 1 is kept the same and the high-affinity substrate 2 is added, Lon will be shifted to the active form, leading to robust degradation of the normally poorly degraded substrate 1.

flagellum and grow a stalk before it can initiate chromosome replication. Thus, the swarmer-to-stalked transition, also known as the G1-to-S transition, is coupled to DNA replication and is intricately linked to regulated protein degradation (56). At the center of this transition is the essential master regulator, CtrA, which is responsible for regulating expression of approximately 100 genes (57–59). CtrA also binds to and inhibits the origin of replication in swarmer cells, preventing swarmer cells from initiating replication. Therefore, when it becomes time to resume DNA replication during the swarmer-to-stalked transition, CtrA must be eliminated. This occurs through dephosphorylation by the CckA pathway (60–62) and through proteolysis by the ClpXP protease (8, 63).

Because the levels of ClpXP remain constant during the cell cycle, additional mechanisms must exist to support the cyclic oscillations seen in CtrA levels during the cell cycle (8). Indeed, a tightly regulated series of events, involving the adaptor proteins CpdR, RcdA, and PopA, and the second messenger cyclic di-GMP ensures the degradation of CtrA (and other substrates) and timely entrance into S phase (64).

Intriguingly, although CtrA degradation in vivo requires ClpXP as well as additional accessory factors, ClpXP can degrade CtrA without these adaptors in vitro (65, 66). This paradox was reconciled by data that showed that CpdR, RcdA, and PopA can increase the rate of CtrA degradation in vitro as the addition of these factors lowered the K_m tenfold, keeping V_{max} constant (66). Intriguingly, the assembly of the adaptors was shown to be essential during times when recognition of CtrA by ClpXP was less robust, such as when CtrA is bound to DNA and thereby inaccessible (67). This ensures that there is complete removal of CtrA to allow resumption of DNA synthesis, as even a small amount of CtrA is enough to inhibit the origin of replication (66, 68).

Regulated protein degradation is linked to cell-cycle progression in other bacteria as well. In most bacteria, FtsZ is an essential component of the cell division machinery. FtsZ polymerization is necessary to form the z-ring, the site where septation and cell division occurs. Studies in *Bacillus subtilis* determined that ClpX inhibits FtsZ polymerization, but in a manner independent of ClpP or ATP hydrolysis (69). However, in *E. coli*, ClpXP has been shown to degrade FtsZ directly, potentially functioning to promote the disassembly of FtsZ polymers (70, 71). Similarly, both ClpAP and ClpXP can degrade *Caulobacter* FtsZ in vitro as well as in vivo (72).

Diversifying Proteolysis Through Hierarchies

Recently, CpdR, PopA, and RcdA have been shown to function as adaptors capable of degrading various classes of substrates in a hierarchical manner in *Caulobacter* (38) (**Figure 4**). The lowest level of the hierarchy consists of ClpXP alone, which can theoretically degrade many substrates limited only by the recognition determinants of those substrates. For example, trapping studies in *E. coli* and *Caulobacter* have identified hundreds of potential substrates, several of which are

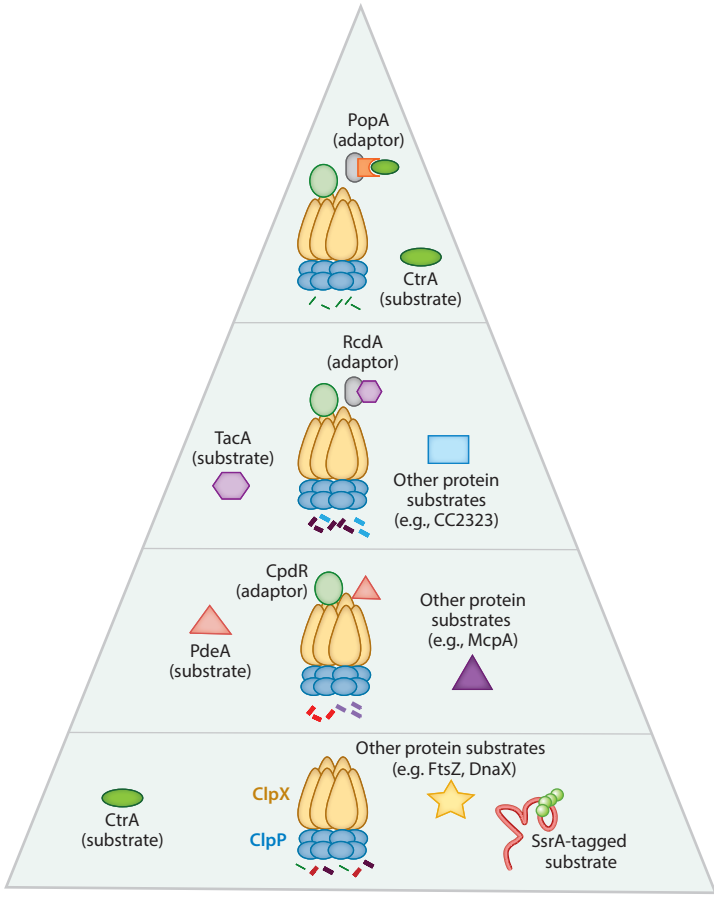


Figure 4

Adaptors assemble in a hierarchical manner to degrade various classes of substrates. ClpXP can degrade numerous substrates on its own, including ssrA-tagged proteins. During the G1-to-S transition in *Caulobacter crescentus*, the adaptor CpdR first primes ClpXP, allowing it to recruit the first class of substrates (PdeA, McpA, etc.) for degradation. The primed protease can now recruit another adaptor, RcdA, to degrade a second class of substrates, such as the transcription factor TacA and others. Finally, the adaptor PopA binds RcdA and in the presence of the second messenger cyclic di-GMP completes the hierarchy to deliver a third class of substrates, including the master regulator CtrA, to ClpXP. As the hierarchy is assembled and adaptors are added onto the protease, specificity increases. When ClpXP is limited, this increase in specificity also comes at the cost of preventing degradation of other members of the substrate pool.

degraded by ClpXP alone in vitro (29, 73), and proteomic studies in *Staphylococcus aureus* illustrate the range of substrate degraded by the Clp family (74). The next tier consists of ClpXP and the adaptor CpdR. Although traditional adaptors can bind their substrates directly, two-hybrid studies found that CpdR alone does not interact with its substrates strongly (37). Instead, CpdR primes ClpXP by binding to the N-terminal domain of the ClpX unfoldase, preparing ClpXP to engage its first class of substrates, which include McpA, a chemoreceptor (62), and the cyclic di-GMP phosphodiesterase PdeA (37, 75). This priming event opens ClpXP to an array of adaptors and substrates that would have been inaccessible before, such as RcdA, which binds a CpdR-primed ClpX directly to establish the third tier of the hierarchy. In this way, RcdA acts as a canonical

adaptor in tethering cargo to ClpXP to enhance delivery of a second class of substrates, including the developmental regulator TacA and various proteins of unknown function (38).

The pinnacle of the hierarchy requires the addition of the adaptor PopA bound to cyclic di-GMP, which culminates in the degradation of CtrA (38, 66, 76) and likely other substrates such as GdhZ and KidO (77, 78). Strikingly, bacterial two-hybrid experiments demonstrated that PopA alleles that cannot engage cyclic di-GMP still bind RcdA (38, 76). As such, even in the absence of delivery, PopA can compete with the RcdA-dependent degradation of substrates such as TacA, suggesting that members of the hierarchy can act as both adaptors and antiadaptors (38, 56). Cross-species comparisons find that CpdR and RcdA are highly conserved in all alpha proteobacteria, but the presence of PopA is more restricted (79), leading to the speculation that CpdR and RcdA could represent a more ancient aspect of this adaptor hierarchy. Interestingly, RcdA is essential in *Agrobacterium tumefaciens* (80) consistent with the ancestral role this adaptor hierarchy plays in the physiology of other bacteria.

The Costs and Benefits of Adaptors

Balanced growth requires regular predictable changes in protein levels to drive replication and division. Although AAA+ proteases can have different ranges of selectivity, there is a clear need to augment their specificity through other regulators. Adaptor proteins in their most basic form can be thought of as simple tethers that locally increase the concentration of potential protease substrates. However, there is a conflict in that adaptors that bind cargo weakly may not efficiently deliver substrates to the protease, whereas adaptors that grip cargo too tightly could also hinder the substrate degradation by the protease. Therefore, there must be an appropriate tuning of the lifetimes of the different subcomplexes involved in adaptor-dependent handoff to ensure robust degradation. Below, we discuss how the well-characterized SspB/ssrA system demonstrates this principle.

As described previously, the SspB adaptor binds ssrA-tagged substrates and delivers them to the ClpXP protease. Bulk measurements find that GFP tagged with ssrA binds SspB with a k_{on} of $\sim 5\ \mu M^{-1}s^{-1}$ at 30°C and a K_D of ~ 50 nM, yielding a ~4-s lifetime for this complex (81) (**Figure 5**). Single-turnover experiments suggest that the limiting step for degradation by ClpXP is substrate commitment, rather than translocation or proteolysis, with an estimate of ~30 s for tagged GFP at saturating concentrations (82). Therefore, cargo can be released from the adaptor's grip seven to eight times during the time that ClpXP is establishing commitment. However, if a cargo binds SspB tenfold more tightly, with the same on rate, then this new 40-s lifetime exceeds the ClpXP commitment time and may result in paradoxically slower proteolysis. Although such substrates have not yet been identified, this simple example illustrates the point that tighter binding of an adaptor to cargo may not result in more robust delivery to the protease and that an optimum balance likely exists between adaptor-cargo lifetimes and protease commitment timescales.

Adaptors can also serve as more than tethers. In addition to anchoring cargo to the protease, adaptor binding may also cause degrons in the cargo protein to be exposed, as described for YjbH/Spx (83) and RssB/RpoS (84, 85). Adaptors could also affect the protease itself. As described above, CpdR binds to ClpX and activates the ClpXP protease for degradation of the substrate PdeA, but CpdR on its own fails to bind PdeA (37). Binding of CpdR, or similar adaptors, may contribute to substrate engagement directly, e.g., providing additional low affinity contacts, or may affect ClpXP substrate engagement through allosteric changes of ClpX itself. Given our discussion above regarding the balance of protease commitment and adaptor-cargo lifetimes, it is tempting to consider that some adaptors may influence protease specificity through altering the commitment time of the protease rather than changing its ability to directly recognize a target.

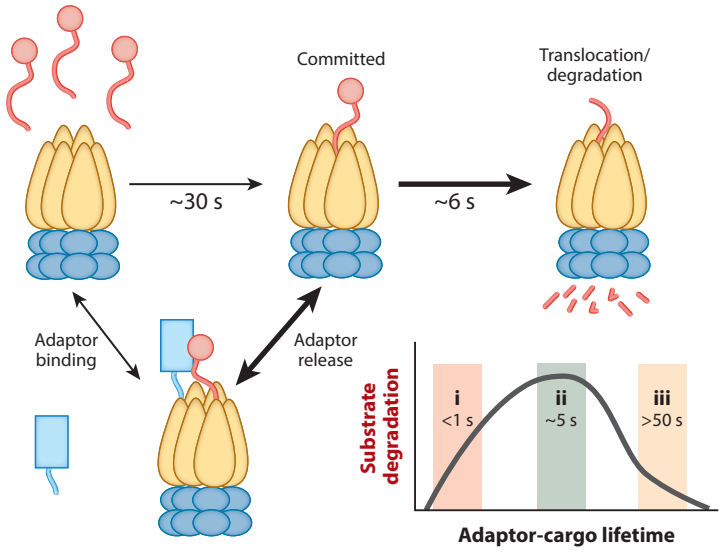


Figure 5

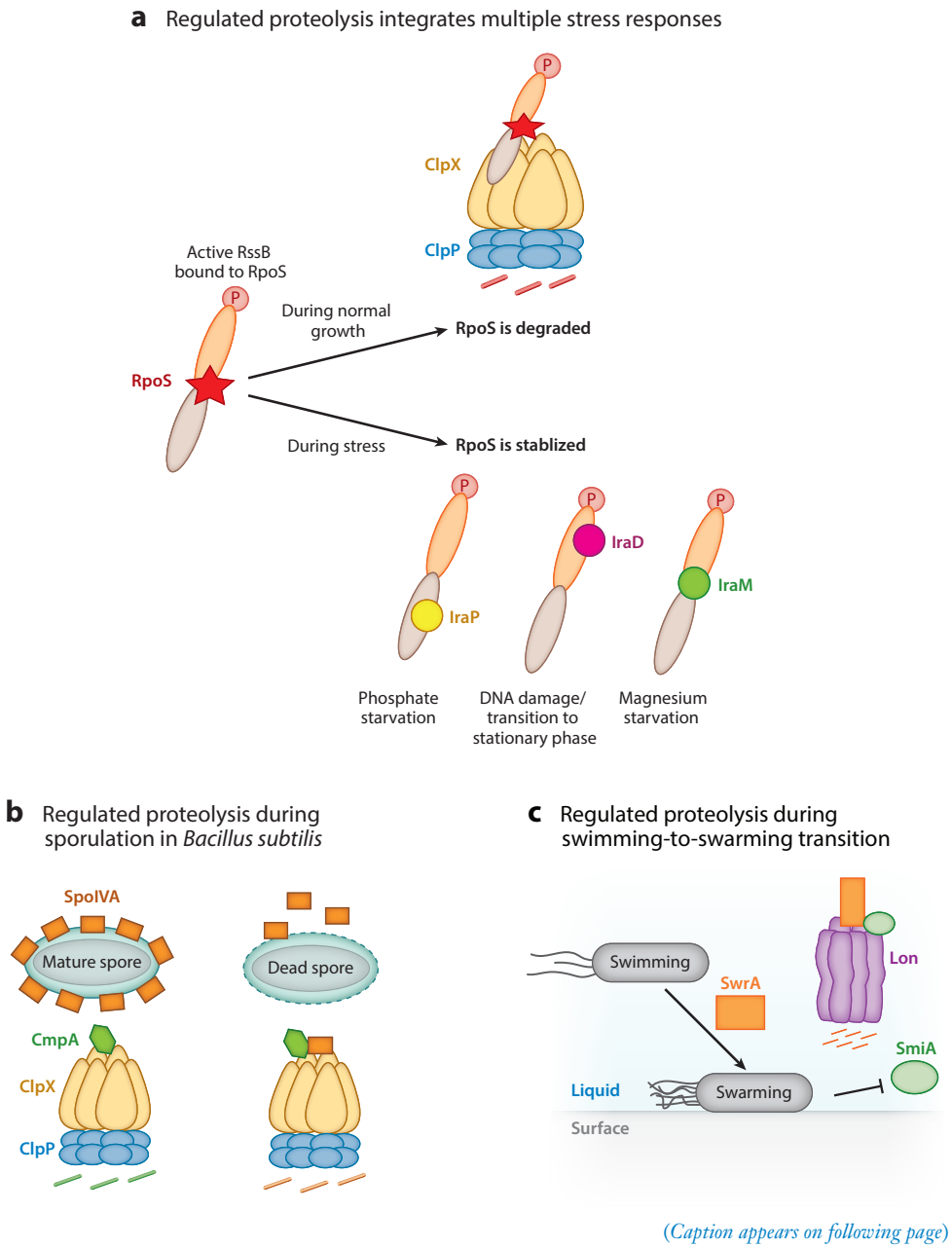
Substrate degradation by ClpXP is rate-limited by the commitment step, where the protease initially engages a target, rather than the unfolding or translocation steps, which are relatively fast. Commitment is estimated to be ~30 s for degradation of tagged GFP by ClpXP (82). Tethering adaptors (such as SspB and RcdA) enhance degradation of substrate, but the strength of the interaction between the adaptor and substrate must be tuned to the commitment time for the protease (*ii*). Poor adaptor-cargo binding results in failure to deliver (*i*), but binding too tightly (*iii*) hinders substrate release during the commitment step for protease engagement of the substrate.

Regulated Proteolysis During Changes in Growth Phase

Bacteria are constantly being challenged in their environments with changing conditions, such as nutrient deprivation, that necessitate a swift response. Regulated proteolysis is required for bacteria to undergo the necessary physiological transitions and adaptation needed for survival and persistence. An example of this is the transition from logarithmic growth to stationary phase, when growth slows and cells alter their metabolism to accommodate this change in phase (86). This transition requires σ^S, also known as RpoS, an alternative sigma factor that can compete with σ^{70} during stationary phase to significantly alter gene expression profiles. During logarithmic growth, ClpXP, with the required assistance of the adaptor RssB, rapidly degrades RpoS to undetectable levels (85, 87) (**Figure 6*a***).

During entry to stationary phase, RpoS becomes stabilized and produces a surge in transcription of RpoS-regulated genes (88, 89). A group of antiadaptors, each specific for a particular stress response, accomplishes inhibition of RssB activity. In *E. coli*, three antiadaptors have been identified: IraP (inhibitor of RssB activity during phosphate starvation), IraD (inhibitor of RssB activity during DNA damage), and IraM (inhibitor of RssB activity during magnesium starvation). Interestingly, each antiadaptor binds to RssB in a different binding mode (2, 90, 91). IraP binds at the C-terminal domain of RssB, IraD interacts with the N-terminal domain, and IraM interacts with both domains. This example shows how a family of distinct antiadaptors, each binding at a characteristic location, can prevent the degradation of RpoS, allowing the cell to mount a rapid universal program in response to a variety of stresses.

In nutrient-poor conditions, *Bacillus subtilis* initiates a sporulation program resulting in mature spores that can withstand harsh environmental conditions. The structural protein SpoIVA is required for proper assembly of the spore envelope. To ensure the fidelity of the sporulation program, *Bacillus* relies on regulated proteolysis as a quality-control mechanism to remove spore envelopes that have been improperly assembled (92) (**Figure 6*b***). In sporulation-defective cells, CmpA, an adaptor, delivers SpoIVA to ClpXP for degradation, ultimately leading to the lysis and



(Caption appears on following page)

Figure 6 (*Figure appears on preceding page*)

Regulated proteolysis is required during physiological transitions and changes in growth. (*a*) When bacteria are actively growing in logarithmic phase, ClpXP rapidly degrades the alternative sigma factor RpoS in an RssB-mediated manner. When RssB is phosphorylated, it has high affinity for RpoS and can deliver it to ClpXP for degradation. Anti-adaptors bind to RssB in different binding modes that depend on the kind of stress the bacteria encounters, preventing it from delivering RpoS for degradation. (*b*) *Bacillus subtilis* requires proteolysis by ClpXP to ensure proper spore envelope assembly. In cells with improperly assembled envelopes, the adaptor CmpA delivers SpoIVA to ClpXP for degradation, leading to lysis of the defective cell. If the spore envelope is properly assembled, the adaptor is targeted for degradation by ClpXP instead. (*c*) Lon-mediated degradation is required for proper motility during the transition from liquid to solid media. In liquid media, Lon degrades SwrA with the help of SmiA, an adaptor protein. Upon shift to solid media, degradation of SwrA is inhibited, leading to an increase in SwrA levels necessary for swarming on solid media.

removal of the defective cell from the population. However, in cells that properly constructed the spore envelope, CmpA is degraded, preventing the degradation of SpoIVA and allowing cells to continue the process of sporulation.

Bacteria growing in a liquid environment use flagella to swim in three-dimensional space. When shifted to solid media, bacteria transition their mode of motility from swimming to swarming. In *Bacillus subtilis*, this transition requires Lon-dependent degradation of SwrA, a master regulator of flagellar biosynthesis (93) (**Figure 6*c***). When *Bacillus* is in a liquid environment, Lon robustly degrades SwrA in the presence of SmiA, a Lon-specific adaptor protein. Lon was unable to degrade SwrA both in vivo and in vitro in the absence of SmiA. When *Bacillus* transitions to a solid surface, Lon-mediated degradation is inhibited via an unknown mechanism and SwrA levels increase, resulting in increased flagellar density, which is necessary for swarming motility. Intriguingly, SmiA was the first Lon-specific adaptor to be discovered, but more recent work using a Lon trapping approach has led to the discovery of HspQ, a conserved, small heat shock protein, that can also enhance Lon-dependent degradation of known substrates in the gram-negative bacterium *Yersinia pestis* (39). Understanding how Lon activity can be controlled in so many different ways and the consequences of this regulation is an outstanding question clearly worth exploring.

Proteolytic Responses in Response to Starvation

Amino acids are the building blocks of proteins and all organisms must be able to accurately assess amino acid levels to avoid costly interruptions in protein synthesis (94). In times of amino acid starvation, protein degradation could replenish amino acid pools. In eukaryotes, inhibition of the ubiquitin-proteasome system results in a lethal depletion of amino acid pools, which could be rescued by externally supplementing with additional amino acids or reducing translation (95). Earlier studies in *E. coli* found that starvation causes an increase in protein degradation that mirrored the rate of synthesis of new proteins (96). However, very little is currently known about how starvation mechanistically leads to amino acid recycling in bacteria.

In bacteria, amino acid starvation leads to increased cellular levels of the alarmone (p)ppGpp, eliciting what is known as the stringent response (97). Increased levels of (p)ppGpp lead to many downstream effects, effectively allowing the cell to divert resources away from translation and toward amino acid biosynthesis (98). One of the effects of (p)ppGpp accumulation is the inhibition of the enzymes that break down polyphosphate, leading to increased polyphosphate levels (99). Work from Kornberg and colleagues has shown that polyphosphate can stimulate the proteolysis of ribosome subunits and other proteins by the Lon protease (100–102). This has led to the provocative hypothesis that during amino acid stress, activation of the Lon protease via ppGpp/polyphosphate

induction will reduce ribosome pools to slow down translation and replenish pools of amino acids. Although recent work suggests that polyphosphate activation of Lon proteolysis may not be a universal feature for all substrates (103), the ability of regulated proteases such as Lon to contribute to nutrient stress responses to improve survival under starvation conditions is a very appealing notion. Indeed, prior work has shown that loss of energy-dependent proteases in bacteria yields compromised responses to starvation (104, 105).

PERSPECTIVE

Energy-dependent proteases can differ dramatically in architecture and substrate preference. However, a recurring theme is that regulated proteolysis requires two elements for robust controlled substrate degradation. All substrates must contain some type of recognition determinant that can be engaged by the protease to begin unfolding and processing. This determinant can be highly sequence dependent, as in the case of ssrA and ClpXP, or it can be more general, as seen with recognition of hydrophobic residues by Lon. To truly maintain regulation, these determinants are further elaborated by modifying factors such as adaptors that deliver substrates directly or prime the protease for substrate recognition. These modifiers can also be a property of the protein itself, e.g., protein dynamics that cause transient or extended display of residues normally in the hydrophobic core. Finally, substrates themselves can act as modifiers to alter the specific activity of a protease in an effort to mount an effective stress response.

Fundamentally, the kinetics of ATP hydrolysis and resulting conformational changes set the limit for how fast the machines can process their substrates. However, there are additional kinetic considerations that impact the activity of specific proteases. For example, proteases responsible for quality control, such as the Lon protease, survey the stability of many substrate proteins by recognizing normally buried hydrophobic residues that are exposed for a sufficiently long time. In this regard, the lifetime of the exposed, unfolded state, rather than specific sequences, is the determining factor for degradation. Adaptor-dependent delivery can elaborate the normal specificity of a protease by scaffolding substrates to the proteases and driving recognition through increasing local concentration. However, if this adaptor-substrate complex is too persistent, then the protease could fail to engage the substrate successfully and fail to degrade the targets robustly. Finally, proteases can transiently adopt an activated state, such as the case of the Lon protease, where high affinity substrates can bind the protease and cause allosteric changes to a more active form that can then robustly degrade low affinity substrates that are normally poorly recognized. These examples highlight the complexity and interplay between kinetics and thermodynamics of the proteases and their substrates in ultimately determining the cellular balance of proteins.

From a therapeutic perspective, physiological consequences of protease loss are especially apparent during stressful situations, such as when pathogens invade their hosts. Therefore, these proteases would be opportune targets to explore for the development of future antibiotics. Indeed, recent studies have shown that small molecule inhibitors of both Clp and Lon family proteases can be highly efficacious for various bacteria (106–108). Perhaps most intriguing, unconstrained activation of these proteases could be as (or more) toxic to the bacteria than inhibition. As a recent illustration of this possibility, the ClpP activating acyldepsipeptide ADEP was able to eradicate *Staphylococcus aureus* even when cells were tolerant of other antibiotics (109).

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