Design principles of the proteolytic cascade governing the $\sigma^E$-mediated envelope stress response in Escherichia coli: keys to graded, buffered, and rapid signal transduction

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Proteolytic cascades often transduce signals between cellular compartments, but the features of these cascades that permit efficient conversion of a biological signal into a transcriptional output are not well elucidated. $\sigma^E$ mediates an envelope stress response in Escherichia coli, and its activity is controlled by regulated degradation of RseA, a membrane-spanning anti-$\sigma^E$ factor. Examination of the individual steps in this protease cascade reveals that the initial, signal-sensing cleavage step is rate-limiting; that multiple ATP-dependent proteases degrade the cytoplasmic fragment of RseA and that dissociation of $\sigma^E$ from RseA is so slow that most free $\sigma^E$ must be generated by the active degradation of RseA. As a consequence, the degradation rate of RseA is set by the amount of inducing signal, and insulated from the “load” on and activity of the cytoplasmic proteases. Additionally, changes in RseA degradation rate are rapidly reflected in altered $\sigma^E$ activity. These design features are attractive as general components of signal transduction pathways governed by unstable negative regulators.

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Proteolytic cascades are widely used to transduce signals across membranes to enable cells to respond to environmental stress and coordinate processes in different cellular compartments. However, the molecular properties of these cascades that facilitate the desired outputs have rarely been examined. In this work, we examine the individual steps in the protease cascade governing the activity of the $\sigma^E$-mediated envelope stress response in Escherichia coli to determine the construction features of this cascade that facilitate faithful transmission of signal and a rapid output.

$\sigma^E$ directs RNA polymerase to transcribe genes encoding proteins that ensure the synthesis, assembly, and homeostasis of outer membrane porins and lipopolysaccharide, the two major components of the unique outer membrane of Gram-negative bacteria [Dartigalongue et al. 2001; Rezuchova et al. 2003; Rhodius et al. 2006]. Envelope integrity is required under all growth conditions, and $\sigma^E$ is an essential transcription factor [De Las Penas et al. 1997a]. Perturbations in the integrity and protein-folding state of the envelope caused by temperature upshift, chaperone depletion, or accumulation of unassembled porins increase $\sigma^E$ activity; conversely, temperature downshift and/or depletion of porins decrease $\sigma^E$ activity [Mecsas et al. 1993; Hiratsu et al. 1995; Raina et al. 1995; Rouviere et al. 1995; Missiakas et al. 1996; Rouviere and Gross 1996, Ades et al. 2003].

The components of the signal transduction system that control $\sigma^E$ activity are shown in Figure 1. RseA, a membrane-spanning anti-$\sigma$ factor, inhibits $\sigma^E$ activity. The cytoplasmic domain of RseA [RseA1–108] binds to $\sigma^E$ and its periplasmic domain binds to RseB [De Las Penas et al. 1997b, Missiakas et al. 1997]. Interaction of $\sigma^E$ with RseA prevents $\sigma^E$ from binding to RNA polymerase [Campbell et al. 2003]. This inhibitory interaction is re-
cleaved when RseA is degraded via a proteolytic cascade. RseA is cleaved first in its periplasmic domain by DegS and then within its transmembrane region by the RIP protease, RseP, thereby releasing RseA1–108 still bound to σE [Ades et al. 1999; Alba et al. 2002; Kanekura et al. 2002; Walsh et al. 2003; Akiyama et al. 2004]. σE is finally released from this inhibitory interaction when RseA1–108 is degraded by cytoplasmic proteases [Flynn et al. 2004].

The proteolytic cascade degrading RseA is activated by unassembled porins in the envelope compartment of the cell. Porins constitute 2% of all cellular proteins and form trimERIC pores in the outer membrane [Nikaido 1996]. When folding is inefficient, unassembled porins accumulate and their C termini activate DegS to initiate cleavage of RseA [Walsh et al. 2003]. Exposed C termini are an excellent indicator of impaired folding as they are normally sequestered in the porin trimer interface [Cowan et al. 1995]. Free DegS is inactive in cleaving RseA because its catalytic triad is inappropriately positioned. However, when porin C termini bind to the DegS PDZ domain, the catalytic triad is repositioned and cleavage of RseA ensues [Walsh et al. 2003; Wilken et al. 2004]. DegS, RseB, RseA, and the PDZ domain of RseP itself all inhibit RseP cleavage of intact RseA, thereby ensuring that DegS always acts before RseP in the RseA-processing pathway [Kanekura et al. 2003; Bohn et al. 2004; Grigorova et al. 2004]. Since DegS is the only protease that senses the porin signal, obligate sequential cleavage of RseA ensures that the initiation of the proteolytic cascade reflects the status of this inducing signal.

In the present study we dissect the individual steps in the proteolytic cascade degrading RseA. A kinetic description of the degradation of RseA under inducing conditions is shown in Figure 2A. Quantification of the disappearance of full-length RseA [Fig. 1, Step 1] reveals that cleavage by DegS is initiated with a T1/2 of 1 min [Fig. 2B]. An intermediate-sized band (termed RseA-I) [Fig. 2A] corresponds to the initial DegS cleavage product: It is identical in size to the initial DegS cleavage product: It is identical in size to the RseA species [full-length RseA, DegS cleavage product, and RseP cleavage product] using the pulse-chase protocol described in Ades et al. (2003) that is known to disrupt the physiological state of the cells (see Materials and Methods).

A typical gel showing pulse chase data for degradation of RseA under inducing conditions is shown in Figure 2A. Quantification of the disappearance of full-length RseA [Fig. 1, Step 1] reveals that cleavage by DegS is initiated with a T1/2 of 1 min [Fig. 2B]. An intermediate-sized band (termed RseA-I) [Fig. 2A] corresponds to the initial DegS cleavage product: It is identical in size to the RseA species [full-length RseA, DegS cleavage product, and RseP cleavage product] using the pulse-chase protocol described in Ades et al. [2003] that is known to disrupt the physiological state of the cells (see Materials and Methods).

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In the present study we dissect the individual steps in the proteolytic cascade degrading RseA. A kinetic description of the pathway reveals that the initial signal-sensing cleavage step is rate-limiting under all conditions, ensuring that the overall degradation rate of RseA reflects signal status. We find that RseA and σE interact with picomolar affinity, ensuring that free σE is generated almost exclusively by cytoplasmic ATP-dependent proteases that disassemble the complex and degrade RseA1–108. Indeed, enormous resources are invested into clearing RseA1–108 from the cell: Essentially, the entire network of cytoplasmic ATP-dependent proteases participates in this process. We will discuss why the organizational structure of this protease cascade facilitates rapid and sensitive transmission of signal to the output response. Proteolytic cascades govern a variety of processes in diverse organisms [Brown et al. 2000; Weihofen and Martoglio 2003]. We believe that the investigation and subsequent cross-comparison of such pathways will significantly improve our understanding of the principles that underlie these signaling proteolytic cascades.

**Results**

**A kinetic description of the degradation of RseA under inducing conditions**

Initiation of RseA degradation by DegS is relatively slow during steady-state growth [T1/2 ~ 8 min] [Ades et al. 2003], and is rate-limiting under these noninducing conditions, as no intermediates in degradation were observed [Ades et al. 1999; Alba et al. 2002; Kanekura et al. 2002; Akiyama et al. 2004]. Here, we determined whether initiation of RseA degradation is rate-limiting when stress is generated by overexpressing the YYF peptide [Walsh et al. 2003]. We measured the T1/2 of all three RseA species [full-length RseA, DegS cleavage product, and RseP cleavage product] using the pulse-chase protocol described in Ades et al. [2003] that is known to disrupt the physiological state of the cells (see Materials and Methods).
The cytoplasmic domain of RseA binds very tightly to $\sigma^E$.

In principle, $\sigma^E$ could be released from RseA1–108 both by dissociation and by degradation. To investigate the relative contributions of these two processes, we characterized the stability of the complex using RseA1–108, a slightly truncated version of the normal cytoplasmic domain of RseA. This fragment is easy to purify because it is stable in E. coli. It is also sufficient for anti-$\sigma$ activity both in vivo and in vitro (De Las Penas et al. 1997b; Missiakas et al. 1997; Campbell et al. 2003). We used fluorescence anisotropy to measure the dissociation of rhodamine-labeled RseA1–100 from $\sigma^E$. To permit rhodamine labeling, residue E28 was mutated to cysteine, chosen because it was far from the RseA–$\sigma^E$ interface. The anisotropy of rhodamine-labeled RseA1–100E28C was considerably lower than that of the labeled RseA1–100E28C/$\sigma^E$ complex (Fig. 3A). Therefore dissociation of labeled RseA1–100E28C from $\sigma^E$ in the presence of excess unlabeled RseA can be measured by determining the kinetics of the decrease in anisotropy. These experiments revealed that in the presence of a 10-fold excess of unlabeled RseA1–100, there was no decrease in anisotropy during a 2-h time course, indicating that the dissociation rate constant of RseA1–100E28C from $\sigma^E$ was much less than $10^{-4}$ sec$^{-1}$ (Fig. 3B). This very slow dissociation rate is not an artifact of the E28C substitution and/or rhodamine labeling since equivalent results were obtained from the reverse experiment: A 10-fold excess of rhodamine-labeled RseA1–100E28C did not compete for unlabeled RseA1–100 bound to authentic $\sigma^E$ during 2 h of incubation (data not shown). These experiments indicate that the RseA1–100/$\sigma^E$ complex dissociates very slowly in vitro.

To measure the association rate constant between rhodamine-labeled RseA1–100E28C and $\sigma^E$, we followed changes in fluorescence intensity after mixing reactants in a stopped-flow apparatus (Fig. 3C). The determined value of $1.5 \pm 0.2 \times 10^7$ M$^{-1}$ sec$^{-1}$ is close to that expected for a diffusion-limited reaction. This rate constant is neither a consequence of the E28C substitution nor of the labeling: Binding of both unlabeled RseA1–100 and labeled RseA1–100E28C to $\sigma^E$ was confirmed to be the same by measuring changes in anisotropy induced by addition of $\sigma^E$ to labeled RseA1–100E28C premixed at different concentrations with unlabeled RseA1–100 [data not shown]. Based on the measured values of the association and dissociation rate constants, the equilibrium dissociation constant ($K_{d}$) between RseA1–100 and $\sigma^E$ in vitro is very tight (<10 pM). Taken together, the rapid degradation of RseA1–100 [Fig. 2C] compared with the very slow spontaneous dissociation of $\sigma^E$ from RseA1–100 strongly suggests that free $\sigma^E$ is predominantly generated by the ATP-dependent, enzyme-catalyzed process of RseA degradation in vivo.

Multiple ATP-dependent proteases degrade RseA1–108 in vitro

The ATP-dependent unfoldase/protease ClpXP is competent both to disassemble the RseA1–108/$\sigma^E$ complex...
Multiple ATP-dependent proteases participate in RseA<sup>+/−</sup> degradation in vivo

To identify the ATP-dependent proteases active against RseA<sup>+/−</sup> in vivo, we determined whether singly deficient protease strains were defective in degradation of RseA<sup>+/−</sup>. Only disruption of clpX or clpP decreased the rate of degradation of RseA<sup>+/−</sup> [Fig. 5A; data not shown], validating previous data that indicated ClpXP as the major protease degrading RseA<sup>+/−</sup> (Flynn et al. 2004). However, as RseA<sup>+/−</sup> was degraded very rapidly in the absence of ClpXP ($T_{1/2} \sim 1.6$ min) [Fig. 5A], additional proteases must be active in RseA<sup>+/−</sup> degradation in vivo.

We tested strains lacking multiple ATP-dependent proteases to identify the additional proteases participating in RseA<sup>+/−</sup> degradation [Fig. 5A; data not shown]. The rate of degradation of RseA<sup>+/−</sup> in a ΔclpAΔclpX strain was the same as that in a ΔclpX strain, even though formation of both the ClpAP and ClpXP proteases are prevented in this strain [data not shown]. This result is consistent with the similar stabilization of RseA<sup>+/−</sup> in a ΔclpX and a ΔclpP strain [Fig. 5A] and suggests that ClpAP does not play a major role in degrading RseA<sup>+/−</sup> in vivo even though ClpAP was almost as efficient as ClpXP in degrading RseA<sup>+/−</sup> in vitro. In contrast, a ΔclpXΔlon strain showed a reproducibly slower rate of degradation of RseA<sup>+/−</sup> ($T_{1/2} = 2.2$ min) than the ΔclpX strain, suggesting that Lon is second to ClpXP in degrading RseA<sup>+/−</sup>. Although this could be an indirect effect, the fact that Lon degrades RseA<sup>+/−</sup> in vitro suggests that the slower rate of degradation is likely to result from eliminating Lon-mediated turnover of RseA. A ΔclpPΔclpXΔlon strain showed a further and larger decrease in degradation of RseA<sup>+/−</sup> [$T_{1/2} = 3.5$ min]. One interpretation of these results is that when Lon is missing, ClpAP degrades RseA<sup>+/−</sup>; removal of ClpP would prevent this latter degradation. Degradation by ClpAP is consistent with its rapid degradation of RseA<sup>+/−</sup> in vitro. Interestingly, a ΔclpPΔclpXΔlonΔhsUv strain further decreases degradation of RseA<sup>+/−</sup> [$T_{1/2} = 4.0$ min] even though HslUV did not degrade RseA<sup>+/−</sup> in vitro [see below]. A ΔclpPΔclpXΔlonΔhsUvΔclpA strain degrades RseA<sup>+/−</sup> somewhat more slowly [$T_{1/2} = 5.5$ min] [Fig. 5A], possibly because ClpA unfoldase function is removed. With the assistance of cytoplasmic protein unfoldases, FtsH may carry out this residual degradation, a proposition we did not test because ftsH is essential. In summary, successive removal of each ATP-dependent protease decreased the rate of degradation of RseA<sup>+/−</sup> in small incremental steps. Some of these decreases could be an indirect reflection of increased protease load on the remaining proteases. However, the fact that four of the five ATP-dependent proteases degrade RseA<sup>+/−</sup> in vitro suggests that the effect is direct. Taken together, these data make the key point that RseA<sup>+/−</sup> has evolved to be a substrate for many proteolytic machines, and as a consequence, even when the cytoplasmic proteolytic machinery is significantly disabled, RseA<sup>+/−</sup> is still degraded very rapidly.

and to degrade RseA<sup>+/−</sup>; RseA<sup>+/−</sup> alone and in complex with σE were degraded at approximately equivalent rates [Flynn et al. 2004]. We determined whether any other ATP-dependent proteases could perform these reactions in vitro. Except for HslUV, all of the ATP-dependent proteases degraded free RseA<sup>+/−</sup> with the following efficiencies: ClpAP > ClpXP > Lon > FtsH under standard in vitro conditions [Fig. 4]. However, whereas ClpXP degrades RseA<sup>+/−</sup> in the RseA<sup>+/−</sup>/σE complex as efficiently as free RseA<sup>+/−</sup>, ClpAP and Lon degrade RseA<sup>+/−</sup> more slowly than free RseA<sup>+/−</sup> and FtsH hardly degrades RseA<sup>+/−</sup> at all. These results are in accord with the lower unfoldase activity of Lon [A. Matouschek, pers. comm.] and FtsH (Herman et al. 2003). We conclude that four of the five ATP-dependent proteases degrade RseA<sup>+/−</sup> in vitro, and three are also capable of catalyzing the disassembly of the RseA<sup>+/−</sup>/σE complex relatively efficiently.
The role of ATP-independent proteases in RseA\(^{1–108}\) degradation

Given the requirement for disassembly, we have thus far solely considered the role of ATP-dependent proteases in degradation of RseA\(^{1–108}\). However, the crystal structure of RseA\(^{1–100}\) indicates that only the first 66 amino acids of RseA interact with \(\sigma^E\); the remainder is predicted to be unstructured and has been found to have a binding site for the ClpX adaptor protein, SspB (Campbell et al. 2003; Flynn et al. 2004; Levchenko et al. 2005). This large unstructured tail might be a substrate for ClpX. We therefore tested whether ClpX might cleave other portions within the unstructured region of RseA. Although ClpX cleavage site for RseP follows Ala 108; however, RseP cleavage by ATP-independent proteases. The preferred binding site for the ClpX adaptor protein, SspB (Campbell et al. 2003; Flynn et al. 2004; Levchenko et al. 2005). The substrate load for the cytoplasmic proteases unstructured tail might generate a new cleavage site for ClpX. The activity of ClpXP is relatively insensitive to perturbations that result in increased degradation of RseA\(^{1–108}\).

The activity of \(\sigma^E\) is relatively insensitive to the flux of substrates through ClpXP

The substrate load for the cytoplasmic proteases undoubtedly varies with growth conditions. We asked whether \(\sigma^E\) activity was sensitive to substrate load on ClpX, the major protease degrading RseA\(^{1–108}\). Major substrates of ClpX are \(\sigma^E\) and sssA-tagged proteins (Schweder et al. 1996; Gottesman et al. 1998). We tested whether decreasing the flux of these substrate proteins through ClpX increases the rate of degradation of RseA\(^{1–108}\). As endogenous RseA\(^{1–108}\) is not observable on Western blots in wild-type cells due to its rapid degradation, we overproduced RseA\(^{1–108}\) and compared its accumulation in wild-type and mutant strains. Both a \(\Delta ssrA\) strain (which lacks sssA-tagged substrates) and a \(\Delta rssB\) strain (which lacks the protein that targets \(\sigma^E\) to ClpX) accumulated less RseA\(^{1–108}\) than the wild-type strain (Fig. 6A), indicating that degradation of RseA\(^{1–108}\) increased when competing substrates are eliminated.

We next asked whether increased degradation of RseA\(^{1–108}\) observed in \(\Delta ssrA\) and \(\Delta rssB\) strains resulted in altered \(\sigma^E\) activity. Note that in these experiments, strains do not contain the RseA\(^{1–108}\) overexpression construct: All forms of RseA are generated exclusively by the endogenous protease cascade. We find that with or without induction of the YYF peptide, \(\Delta ssrA\) [Fig. 6B, top] and \(\Delta rssB\) [Fig. 6B, bottom] have \(\sigma^E\) activity equivalent to that of the wild-type strain even though RseA\(^{1–108}\) was degraded more rapidly in the mutant strain backgrounds [Fig. 6A]. This result is in agreement with our finding that the initial cleavage step is the slowest in the pathway and shows that this kinetic arrangement makes \(\sigma^E\) activity insensitive to perturbations that result in increased degradation of RseA\(^{1–108}\).

Figure 4. Multiple ATP-dependent proteases degrade RseA\(^{1–108}\) in vitro. Degradation of RseA\(^{1–108}\) alone or RseA\(^{1–108}\) complex with \(\sigma^E\) [RseA\(^{1–108}\)\(\sigma^E\)] by ATP-dependent proteases was carried out as described in Materials and Methods. Samples were removed at the defined time intervals, separated by SDS-PAGE, and stained with Sypro Ruby protein gel stain. Bands were visualized by a FluorImager.
induced ~1.5-fold to twofold less than that of the wild-type strain (Fig. 6C; Flynn et al. 2004). Two factors contribute to lower activity in the \( \texttt{H9004} \texttt{clpX} \) strain. First, initial DegS cleavage is slower than in the wild-type strain (Fig. 6C, inset: \( T_{1/2} = 1.1 \) min for wild type vs. 1.9 min for \( \texttt{H9004} \texttt{clpX} \)), possibly because physiological changes in \( \texttt{H9004} \texttt{clpX} \) cells might indirectly alter membrane proteolysis. Second, degradation of RseA1\( -108 \) in \( \texttt{H9004} \texttt{clpX} \) cells (\( T_{1/2} = 1.6 \) min) is now comparable to the rate of RseA cleavage by DegS (\( T_{1/2} = 1.9 \) min), so that DegS cleavage and degradation by cytoplasmic proteases become colimiting. The net result is a somewhat slower generation of free \( \texttt{H9268} \texttt{E} \). We note that the magnitude of the defect in \( \texttt{H9268} \texttt{E} \) induction is significantly less than the magnitude of the proteolytic defect. Thus, the organization of the proteolytic cascade decreases the sensitivity of \( \texttt{H9268} \texttt{E} \) activity to alterations in the proteolytic environment of the cell that diminish degradation of RseA\( ^{1-108} \).

Discussion

The goal of this study was to investigate the design principles that permit a protease cascade to convert a signal generated in the envelope to an immediate change in the activity of a transcription factor. To this end, we examined individual steps in the proteolytic pathway that
controls the activity of the essential transcription factor $\sigma^E$ by regulating the degradation of the anti-$\sigma$ factor, RseA. Our major findings are that the signal-sensing cleavage step initiating the pathway is rate-limiting under all conditions, that this proteolysis pathway is robust to changes in the activity of cytoplasmic proteases, and that free $\sigma^E$ is released almost exclusively by proteolysis. Below we discuss the construction principles that underlie each of these characteristics and their biological significance.

Organization of the proteolytic cascade

The proteolytic cascade consists of three sequential steps: a ligand-activated, initial cleavage step carried out by DegS, in which RseA is cleaved in the periplasmic domain; a highly regulated (possibly conformationally controlled) second step carried out by the RIP protease, RseP, which releases the RseA$^{1-108}/\sigma^E$ complex from the membrane; and a final disassembly-degradation step, carried out by multiple ATP-dependent proteases within the cytoplasmic compartment of the cell (Figs. 1, 4, 5A; Ades et al. 1999; Alba et al. 2002; Kanehara et al. 2002; Walsh et al. 2003; Akiyama et al. 2004; Dong and Cutting 2003; Zhou and Kroos 2004). This last step releases free $\sigma^E$, which binds to RNA polymerase and promotes transcription of the $\sigma^E$-regulon to combat envelope stress.

This work demonstrates that even under inducing conditions, the signal-sensitive DegS cleavage step is the slowest in the proteolytic cascade ($T_{1/2} = 1$ min); the two subsequent steps are each at least threefold faster than the initiating cleavage step (Fig. 2). This arrangement ensures that the overall rate of degradation of the protein reflects the status of the signal up to DegS cleavage rates as rapid as $T_{1/2} \sim 30$ sec.

DegS-independent cleavage of RseA by RseP (a RIP protease) is highly controlled, whereas full-length RseA is cleaved very slowly by RseP, DegS-processed RseA is cleaved rapidly (Alba et al. 2002; Kanehara et al. 2002). The mechanistic details underlying this are unknown; however DegS, RseB, the periplasmic domain of RseA, and the PDZ domain of RseP interact in at least two independent mechanisms to repress activity of this protease toward full-length RseA (Kanehara et al. 2003; Bohn et al. 2004; Grigorova et al. 2004). We have suggested that RseP cleavage of full-length RseA is blocked so that DegS cleavage of RseA will be rate-limiting over a wide range of OMP signal. Interestingly, other RIP proteases that are widely used to transmit signals via a protease cascade between the compartments of a bacterial cell appear to occupy an intermediate position in every cascade: They transmit the signal rather than initiate the cascade and their activity is inhibited until the initial cleavage event is completed (Schobel et al. 2004; Chen et al. 2005; Matson and DiRita 2005). Cells use widely different proteins to accomplish inhibition; it remains an open question as to whether these inhibitory events are mechanistically similar or distinct (Resnekov and Losick 1998; Dong and Cutting 2003; Zhou and Kroos 2004, 2005). It will be interesting to examine how quantitative relationships between initial and RIP-mediated cleavages further the output goals of several different systems.

Free $\sigma^E$ is released only after degradation of RseA$^{1-108}$. This point in the pathway could provide an opportunity for a second regulatory step: RseA$^{1-108}$ might be treated as a dead-end product to be eliminated at all costs; alternatively, its degradation (and $\sigma^E$ activity) might be modulated in concert with other proteolytic events key to the stress status of the cell; for example, the rate of degradation of $\sigma^E$. To examine this issue, we performed a careful analysis of the proteases degrading RseA$^{1-108}$ and investigated the cellular consequences of increasing or decreasing the rate of degradation of RseA$^{1-108}$. These studies together with other data provide a clear answer: The cell uses multiple mechanisms to ensure that RseA$^{1-108}$ is degraded regardless of the proteolytic status of the cell. First, RseA$^{1-108}$ is the most rapidly degraded substrate of its major protease, ClpXP ($T_{1/2} \approx 20$ sec) (Fig. 2C). Thus, DegS cleavage will remain rate-limiting even if competing substrates slightly decrease its rate of degradation by ClpXP. Second, in the unlikely event that ClpXP is overwhelmed by other substrates, RseA$^{1-108}$ is degraded rapidly by other proteases ($T_{1/2} = 1.6$ min), leaving DegS cleavage of RseA rate-limiting under a broad range of conditions and thereby ensuring minimal effects on $\sigma^E$ activity (Fig. 6C). Finally, the two major proteases that degrade RseA$^{1-108}$, ClpXP and Lon, are both in the $\sigma^E$-regulon (Rhodius et al. 2006). Hence, under conditions inducing $\sigma^E$, the amount of these proteases will increase to further ensure that DegS cleavage remains rate-limiting. It will be interesting to determine whether uncoupling of degradation rate from the proteolytic status of cytoplasmic proteases is a general characteristic of systems regulated by unstable anti-ors.

RseA$^{1-108}$ is the first protein identified to be a substrate of all of the ATP-dependent proteases. This allowed us to examine the interconnections between these proteases. Our most important finding was that ClpAP degrades RseA$^{1-108}$ very poorly in vivo even though it degrades both free and $\sigma^E$-bound RseA$^{1-108}$ almost as well as ClpXP in vitro. The low activity of ClpAP in vivo cannot be explained by ClpS or SspB inhibition of ClpAP (Flynn et al. 2001; Dougan et al. 2002; Farrell et al. 2005) as ClpAP still has very little activity against RseA$^{1-108}$ in ΔclpS, ΔsspB and ΔclpSΔsspB strains [R. Chaba, unpubl.; data not shown]. Possibly, a currently unidentified inhibitor may prevent ClpAP from degrading RseA$^{1-108}$ in vivo. Additionally, our data raises the possibility that HslUV degrades RseA$^{1-108}$ in vivo, but not in vitro. The simplest explanation for this discrepancy is that ATP-independent protease(s) expose an HslUV-degradation tag, more complex explanations, such as an unidentified HslUV-adaptor protein needed for substrate recognition, can be envisioned. Our study suggests that RseP might be a candidate protease to expose an HslUV recognition signal. Previous studies indicated that RseP cleaves RseA only at a single position, both in vivo and in vitro, which resulted in generation of RseA$^{1-108}$. However, these studies were performed on an artificial substrate, with
both cytoplasmic and periplasmic domains replaced by entities more amenable to analysis [Akiyama et al. 2004]. RseA<sup>1–108</sup> has a long extended tail; it is possible that RseP cleaves at additional positions within this tail or at other unstructured regions of the protein under conditions where degradation of RseA<sup>1–108</sup> is delayed (Campbell et al. 2003). We plan to test this idea as it would be the first indication that a RIP protease can act on the cytoplasmic portion of a protein.

Transducing the degradation signal to a change in activity of σ<sup>E</sup>

σ<sup>E</sup> and RseA<sup>1–100</sup> bind extremely tightly; their picomolar affinity is comparable to that of an antigen–antibody interaction. This exceptionally tight binding is explained by the extensive interface between the two proteins: RseA<sup>1–100</sup> is sandwiched between the two domains of σ<sup>E</sup>, making extensive interactions with each domain, and burying a total surface area of 3805 Å<sup>2</sup> [Campbell et al. 2003]. To dissociate spontaneously, σ<sup>E</sup> would need to break all of these interactions, a situation that rarely occurs. Instead, σ<sup>E</sup> is released by an extremely efficient disassembly-degradation reaction catalyzed by the cytoplasmic ATP-dependent proteases, which occurs with a <i>T</i><sub>1/2</sub> of <20 sec.

Why does the cell use the energy intensive process of disassembly and degradation of RseA<sup>1–108</sup> to set the rate of σ<sup>E</sup> release directly rather than relying solely on indirectly regulating free σ<sup>E</sup> by changing the relative levels of RseA and σ<sup>E</sup>? This latter scenario avoids the high-energy cost of dissociating the two proteins because it does not require very tight binding between σ<sup>E</sup> and RseA. Modeling kinetics of down-regulation of σ<sup>E</sup> activity in each scenario, as demonstrated in Figure 7, provides a possible answer. σ<sup>E</sup> down-regulation results from a decrease in the DegS activating signal, which translates into decreased DegS cleavage of RseA, such as occurs after temperature downshift [Ades et al. 2003]. In a dissociation-dependent scenario, free σ<sup>E</sup> (and thus σ<sup>E</sup> activity) is set by the relative levels of RseA and σ<sup>E</sup>. RseA levels rise slowly in response to its decreased degradation [Fig. 7A, top]; free σ<sup>E</sup> drops in concert with the rise in RseA level [Fig. 7A, bottom], resulting in a slow decrease in σ<sup>E</sup> activity. In contrast, when free σ<sup>E</sup> is generated solely by proteolysis, RseA stabilization results in a rapid decrease in free σ<sup>E</sup> [Fig. 7B, bottom] prior to any change in the relative levels of RseA and σ<sup>E</sup> [Fig. 7B, top] provided that RseA is at least stoichiometric with σ<sup>E</sup>. Intuitively, the rapid drop in free σ<sup>E</sup> is explained by the fact that the rate of σ<sup>E</sup> release would slow immediately upon a decrease in RseA degradation, while any preexisting σ<sup>E</sup> rapidly reassociates with RseA [Fig. 3C]. Published data and our unpublished results suggest that RseA is in excess over σ<sup>E</sup> under basal conditions (Ades et al. 2003) and the high rate of synthesis of RseA relative to σ<sup>E</sup> under inducing conditions (Ades et al. 2003) suggests that this scenario is possible under inducing conditions as well. Results from this scenario fit well with experimental determinations, indicating that σ<sup>E</sup> exhibits a 10-fold decrease in activity within a few minutes after temperature downshift, at a time when the RseA::σ<sup>E</sup> ratio is virtually unchanged from its value at high temperature [Ades et al. 2003]. This situation obtains because RseA stabilization is compensated by decreased synthesis from its σ<sup>E</sup>-dependent promoter. Likewise, in accordance with the proteolysis-controlled model, upon temperature upshift, increase in σ<sup>E</sup> activity occurs prior to change in the RseA::σ<sup>E</sup> ratio [I. Grigorova, unpubl.; data not shown]. Only extreme inducing signals, such as continuous overexpression of outer membrane porins, lead to a significant change in the RseA::σ<sup>E</sup> ratio, possibly because degradation overwhelms synthesis [Ades et al. 1999]. In conclusion, physiological inducing signals that trigger DegS-mediated cleavage of RseA, work by increasing the dynamics of RseA production and degradation and therefore the dynamics of binding and release of σ<sup>E</sup>, rather than by rapidly changing the level of RseA.

![Figure 7](image_url)

**Figure 7.** Two scenarios by which stabilization of RseA could decrease σ<sup>E</sup> activity. (A) An increase in the level of RseA relative to that of σ<sup>E</sup> decreases σ<sup>E</sup> activity. [Top] Analytical calculation of the change in RseA level resulting from changing its rapid degradation at 43°C (T<sub>1/2</sub> = 2 min) to its slow degradation at 30°C (T<sub>1/2</sub> > 50 min) [Ades et al. 2003], reveals that the characteristic time of RseA increase upon temperature downshift is on the order of tens of minutes. [Bottom] Thus, a decrease in the level of free σ<sup>E</sup> (σ<sup>E</sup> not bound to RseA), which determines σ<sup>E</sup> activity, occurs very slowly in concert with the rise in RseA. (B) A decrease in the rate of σ<sup>E</sup> release from RseA decreases σ<sup>E</sup> activity. This scenario requires that the degradation rate of RseA >> the dissociation rate of RseA from σ<sup>E</sup>. Computer simulation of a quantitative model of our system using parameters determined experimentally in this work and Ades et al. (2003) [RseA degradation, σ<sup>E</sup> and RseA synthesis, dilution by cell growth, and binding] [data not shown] demonstrated that decreased release of σ<sup>E</sup> into the cytoplasm as a consequence of decreased initiation of RseA degradation by DegS would result in rapid down-regulation of free σ<sup>E</sup> [bottom] on a time scale much faster than changes in the relative levels of the two proteins [top]. This scenario does not require changes in the relative levels of σ<sup>E</sup> and RseA.
The tight coupling of proteolysis and activity found in the RseA/σE pathway might be important for other Group 4 (ECF) σs in addition to σE. Group 4 σs comprise the majority of the alternate σs, and they control a wide range of responses to environmental stress in diverse bacteria. A predominant paradigm is that these ECF σs positively control their own transcription and are located in operons together with their anti-σs (Missiakas and Raina 1998; Raivoio and Silhavy 2001; Bashyam and Hasnain 2004). These anti-σs are often regulated by proteolysis and show increased degradation when stress increases and decreased degradation when stress decreases (Browning et al. 2003; Schobel et al. 2004; Reiling et al. 2005). We suspect that the ability to immediately down-regulate activity in response to decreased proteolysis will be an important design principle for the proteolytic cascades governing the activity of many Group 4 σs and possibly in other systems governed by unstable negative regulators.

Materials and methods

Media, antibiotics, strains, and plasmids

Luria-Bertani (LB) and M9 minimal medium were prepared as described (Sambrook et al. 1989). M9 was supplemented with 0.2% glucose, 1 mM MgSO4, 2 µg/mL thiamine, and all amino acids (40 µg/mL), except methionine. When required, the medium was supplemented with 100 µg/mL ampicillin (Ap), 20 µg/mL chloramphenicol (Cm), 30 µg/mL kanamycin (Kan), 50 µg/mL spectinomycin (Spec), and/or 10 µg/mL tetracycline (Tet). Expression of proteins from T7, Ptrc, or mutated Ptrc promoters was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Table 1. Strains and plasmids used in this study

| Strains/plasmids | Relevant genotype | Source/reference |
|------------------|-------------------|------------------|
| Strains          |                   |                  |
| BL21(DE3)        | F' ompT gal dcm lon hsdS8[rfa- mCR'] ΔDE3 | Studier and Moffatt 1986 |
| MC1061           | araD ara-lev/7697 Δ(codB-lacI) galK16 galE15 mcrA0 relA1 rpsL150 spoT1 mcrB9999 hsdR2 | Casadaban and Cohen 1980; E. coli Genetic Stock Center |
| MG1655           | rph-1             |                  |
| CAG16037         | MC1061 [ΔaffG]P3::lacZ | Guyer et al. 1981; Jensen 1993; E. coli Genetic Stock Center |
| CAG45114         | MG1655 [ΔaffG]P3::lacZ | Ades et al. 2003 |
| CAG43450         | 16037 pBA166, ApR | Walsh et al. 2003 |
| CAG53072         | 44350 rssB::kanR, KanR ApR | This work |
| CAG53073         | 44350 ssrA::kanR, KanR ApR | This work |
| CAG53077         | 45114 pBA166, ApR | This work |
| CAG53078         | 53077 clpX::kanR, KanR ApR | This work |
| CAG53092         | 45114 pRC8, ApR | This work |
| CAG53144         | 53092 rssB::kanR, KanR ApR | This work |
| CAG53153         | 16037 pRC8, ApR | This work |
| CAG53162         | 53153 ssrA::kanR, KanR ApR | This work |
| CAG53193         | 45114 clpX::kanR pRC14, KanR ApR | This work |
| CAG53194         | 45114 clpP::cmr pRC14, CmR ApR | This work |
| CAG53195         | 53193 lon::tetR, KanR TetR ApR | This work |
| CAG53206         | 53193 rseP::specR, KanR SpecR ApR | This work |
| CAG53209         | 45114 pRC14, ApR | This work |
| CAG53215         | 53215 rseP::specR, KanR TetR SpecR ApR | This work |
| CAG53217         | 45141 clpPclpXlon::cmr pRC14, CmR ApR | This work |
| CAG53218         | 53217 clpA::kanR, CmR KanR ApR | This work |
| CAG53220         | 53217 hslUV::tetR, CmR TetR ApR | This work |
| CAG53228         | 53218 hslUV::tetR, CmR KanR TetR ApR | This work |
| Plasmids         |                   |                  |
| pBA166           | YYF peptide in pTrc99a, ApR | Walsh et al. 2003 |
| pTE6             | his6-RseA1–100 in pET28b, KanR | This work |
| pPER76           | his6-ωE in pET15b, ApR | Rouviere et al. 1995 |
| pRC8             | RseA1–108 in pBA169, ApR | This work |
| pRC10            | –10 box of Prc10 changed to Plac in pBA169, ApR | This work |
| pRC14            | RseA1–108 and ωE in pRC10, ApR | This work |
| pRseA1–100E28C   | his6-RseA1–100E28C in pET28b, KanR | This work |
| pET15b           | Vector, pBR322 ori, ApR | Novagen |
| pET28b           | Vector, pBR322 ori, KanR | Novagen |
| pTrc99a          | Vector, pBR322 ori, ApR | Amersham Pharmacia Biotech |
| pBA169           | pTrc99a ΔNcoI, ApR | Walsh et al. 2003 |
Strains and plasmids are listed in Table 1. Most experiments used MG1655; comparable results were obtained with MC1061. Details of plasmid construction are available on request.

**Buffers**
Buffer I comprises 10 mM Tris-HCl (pH 9.0), 5% glycerol, 0.1 mM aminothiobenzensulfonic acid (AEBSF), and 0.5 mM β-mercaptoethanol (BME).

PD buffer contains 25 mM HEPES-KOH (pH 7.5), 5 mM KCl, 5 mM MgCl₂, 0.02% NP-40, and 10% glycerol. HO buffer contains 50 mM HEPES-KOH (pH 7.5), 20 mM MgCl₂, 300 mM NaCl, 10% glycerol, and 0.5 mM DTT. Buffer H consists of 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 25 µM zinc acetate, 0.1% NP-40, 10 mM MgCl₂, and 1 mM DTT. Lon buffer contains 50 mM Tris-HCl (pH 8.0), 5 mM KCl, 15 mM MgCl₂, and 2 mM DTT.

**Proteins**
ClpA (Maurizi et al. 1994), ClpP (Kim et al. 2000), ClpX (Levchenko et al. 1997), Lon (Goldberg et al. 1994), RseA1 (Levchenko et al. 1997), FtsH (Herman et al. 2003), HslU, HslV, ClpA (Maurizi et al. 1994), ClpP (Kim et al. 2000), ClpX (Maurizi et al. 1994), and Lon (Goldberg et al. 1994), were transformed in BL21(DE3). Cultures were grown at 37 °C in LB containing appropriate antibiotics to an O.D. 600 ∼ 0.5 and induced with IPTG for 1 h. Harvested cells were subjected to induction with IPTG for 1 h; harvested cells were subjected to -100 and 100 were diluted to appropriate concentration using dialysis buffer without glycerol. The samples were degassed under vacuum at room temperature before data collection. Data was collected with a K2 Multifrequency Fluorimeter [ISS] set to λ_ex = 543 nm and λ_em = 575 nm.

The binding curve of labeled his6-RseA1–100E28C and his8-RseA1–100E was obtained by measuring polarization anisotropy for 100 nM labeled his8-RseA1–100E in the presence of various concentrations of his6-RseA. Relative affinities of labeled his8-RseA1–100E28C and unlabeled his8-RseA1–100 were measured by adding different concentrations of his8-RseA to the mixture of labeled and unlabeled proteins [100 nM each protein]. Both the proteins bound his8-RseA with the same affinity. The dissociation rate of labeled his8-RseA1–100E28C/his8-RseA1–100 complex [100 nM] was measured by monitoring anisotropy of the complex upon addition of excess unlabeled his8-RseA1–100 [1.5 µM]. The dissociation rate of unlabeled his8-RseA1–100/his8-RseA1–100 complex was measured in a similar manner in the presence of excess labeled his8-RseA1–100E28C.

**Kinetic assay**
The time course of his8-RseA1–100E28C was obtained by following the increase in rhodamine fluorescence intensity (λ_ex = 543 nm, λ_em = 575 nm) upon formation of the complex, using a stopped flow hand-operated mixing system (SFA-20, HiTech Scientific) and fluorimeter (K2, ISS). To calculate the association rate constant of his6-RseA1–100E28C, the time course of their binding was measured for various concentrations of the two proteins (125 nM and 128 nM, 247 nM and 128 nM, 250 nM and 100 nM, 128 nM and 370 nM of his6-RseA1–100 and labeled his6-RseA1–100E28C). The data was analyzed using Kaleidagraph (Synergy Software) by fitting to the function shown in Equation 1.

\[
I = I_0 + \Delta I \cdot C
\]

where \(I_0\) is the fluorescence intensity of labeled his8-RseA1–100E28C not in complex with his6-RseA1–100E28C and \(\Delta I\) is the increase in fluorescence intensity upon his8-RseA1–100E28C binding, \(C\) is the fraction of labeled his8-RseA1–100E28C in complex with his6-RseA1–100E28C and \(k\) is the association rate constant.

**Determination of RseA, RseA-I, and RseA 1–108 stability by pulse-chase immunoprecipitation**
Cells were grown in supplemented M9 minimal medium lacking methionine [with 100 µg/mL Ap] at 30°C to an O.D. 600 of 0.15-0.25. Cultures were induced with IPTG for 10 min to overexpress YYF peptide or RseA1–108/his8-RseA1–108. Cells were pulse-labeled with 15 sec with L-[35S] methionine followed by a chase of 1% unlabeled methionine. Samples were processed as described (Ades et al. 2003). As an internal standard, an equal amount of extract from L-[35S] methionine-labeled cells overexpressing the truncated form of RseA, HA-RseA140 [also designated as RseA* in the text] (Kanekura et al. 2002) was added to each sample prior to immunoprecipitation. Samples were immunoprecipitated with antibodies against the cytoplasmic domain of RseA. The time course of RseA-I, both accumulation and disappearance of this intermediate on the gel was quantified. The net generation of RseA-I resulting from RseA cleavage by DegS and its disappearance upon cleavage by RseP can be described by Equation 2, where \(k_{DegS}\) and \(k_{RseP}\) are the rate cont-
stains of RseA cleavage by DegS and RseA-I cleavage by RseP, respectively.

\[ RseA - I = \frac{k_{DegS}}{k_{RseP} - k_{DegS}} \times \left( \exp \left( -k_{DegS}t \right) - \exp \left( -k_{RseP}t \right) \right) \]  

(Eq. 2)

Maximal accumulation of RseA-I was measured to be between 25 and 50 sec after the chase. Using the value for \( k_{DegS} \) determined as described (Ades et al. 2003) and Equation 3, we have estimated \( k_{RseP} \) and thus the \( T_{1/2} \) of RseA-I.

\[ \left( k_{RseP} - k_{DegS} \right) \cdot t_{\text{max}} = \ln \left( \frac{k_{RseP}}{k_{DegS}} \right) \]  

(Eq. 3)

\( T_{1/2} \) of RseA-I can be also estimated from the ratio of RseA-I to RseA that does not change with time at longer time points. For this, we quantified the ratio of RseA-I to RseA at each time point and calculated \( T_{1/2} \) of RseA-I using Equation 4.

\[ \frac{RseA - I}{RseA} = \frac{k_{DegS}}{k_{RseP} - k_{DegS}} \times \left[ 1 - \exp \left( k_{DegS} - k_{RseP} \right) \cdot t \right] \]  

(Eq. 4)

The \( T_{1/2} \) of RseA-I measured using Equation 4 was in good agreement with that measured using Equations 2 and 3.

### Degradation assays

In order to monitor the degradation of RseA\(^{1-108} \), ATP-dependent proteases [ClpX; 0.3 mM and ClpP; 0.8 µM for ClpXP; ClpA; 0.3 µM and ClpP; 0.8 µM for ClpAP; HisLU; 0.3 µM and HisLV; 0.8 µM for HisLU; Lon; 0.3 µM, FtsHc; 0.3 µM, ATP [4 mM] and an ATP regeneration system (50 µg/mL creatine kinase and 2.5 mM creatine phosphate) were mixed in the appropriate buffer and incubated for 2 min at either 30°C or 37°C. RseA\(^{1-108} \) [2 µM] or RseA\(^{1-108}/\sigma^E \) (the complex was formed by incubating 2 µM of RseA\(^{1-108} \) with 4 µM \( \sigma^E \)) was added to initiate the reaction. Samples were removed at various intervals, electrophoresed on SDS-PAGE, and visualized using Sypro Ruby protein gel stain (Molecular Probes) on a FluorImager 595 [Molecular Dynamics].

Degradation assays with ClpAP and ClpXP were performed at 30°C, whereas for FtsH, HisLU, and Lon the reactions were carried out at 37°C. Buffer condition was HO buffer for ClpAP and ClpXP, PD buffer for ClpXP and HisLU, Buffer H for FtsH, and Lon buffer for Lon.

### Western blotting

The level of RseA\(^{1-108} \) and \( \sigma^E \) was monitored by Western blot analysis. Cultures were grown in LB [with 100 µg/mL Ap] at 30°C to an O.D.\(_{600} \) = 0.3 and induced with IPTG for 1 h. Samples were processed as described (Alba et al. 2001) and transferred to PVDF membrane. The following dilutions of primary antisera (all rabbit) were used: anti-RseA cytoplasmic domain (1:10,000) and anti-\( \sigma^E \) (1:10,000). The secondary antibody (used at 1:10,000) was an anti-rabbit HRP [Amersham Life Sciences]. Blots were developed with SuperSignal West Dura Extended Duration Substrate [Pierce]. Epi Chemi Ix Darkroom (UVP Laboratory Products) was used to capture the light emitted from the blots. The intensity of the bands was quantified using associated software (Labworks).

### \( \beta \)-Galactosidase assays

Overnight cultures were diluted to an O.D.\(_{600} \) of 0.015 in LB with 100 µg/mL Ap and grown at 30°C to an O.D.\(_{600} \) of 0.1–0.15. Cultures were induced with IPTG to overexpress YFP peptide. \( \sigma^E \) activity was measured by monitoring \( \beta \)-galactosidase expression from a single-copy \( \sigma^E \)-dependent lacZ reporter gene as described (Miller 1972; Meesas et al. 1993; Ades et al. 1999). All assays were performed three times to ensure reproducibility of the data.

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Design principles of the proteolytic cascade governing the $\sigma^E$-mediated envelope stress response in *Escherichia coli*: keys to graded, buffered, and rapid signal transduction

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