Degradation of the E. coli antitoxin MqsA by the proteolytic complex ClpXP is regulated by zinc occupancy and oxidation

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It is well established that the antitoxins of toxin–antitoxin (TA) systems are selectively degraded by bacterial proteases in response to stress. However, how distinct stressors result in the selective degradation of specific antitoxins remain unanswered. MqsRA is a TA system activated by various stresses, including oxidation. Here, we reconstituted the Escherichia coli ClpXP proteolytic machinery in vitro to monitor degradation of MqsRA TA components. We show that the MqsA antitoxin is a ClpXP proteolysis substrate, and that its degradation is regulated by both zinc occupancy in MqsA and MqsR toxin binding. Using NMR chemical shift perturbation mapping, we show that MqsA is targeted directly to ClpXP via the ClpX substrate targeting N-domain, and ClpX mutations that disrupt N-domain binding inhibit ClpXP-mediated degradation in vitro. Finally, we discovered that MqsA contains a cryptic N-domain recognition sequence that is accessible only in the absence of zinc and MqsR toxin, both of which stabilize the MqsA fold. This recognition sequence is transplantable and sufficient to target a fusion protein for degradation in vitro and in vivo. Based on these results, we propose a model in which stress selectively targets nascent and zinc-free MqsA, resulting in exposure of the ClpX recognition motif for ClpXP-mediated degradation.

Bacterial toxin–antitoxin (TA) modules are ubiquitous genetic elements that code for a toxin capable of inhibiting bacterial growth and a cognate antitoxin that binds and inhibits toxin activity (1–4). For more than a decade, it has been hypothesized that TA systems play a key role in bacterial persistence, a phenotypic state of bacteria characterized by growth arrest and increased resistance to antibiotics. Consistent with this hypothesis, multiple studies have confirmed links between TA systems and persistence, including gene expression profiling experiments that identified TA toxins as the most highly upregulated genes in isolated Escherichia coli persistor cells (5–7). However, recent work has shown that, under the conditions tested, deleting the TA systems in E. coli does not significantly contribute to bacterial fitness, even upon exposure to stress, suggesting that the presence of TA systems may not significantly promote survival (8–10). Despite their prevalence throughout the bacterial kingdom, the endogenous roles of TA systems and how they contribute to bacterial fitness are still poorly understood.

Although the biological functions of TA systems remain elusive, how they function and are regulated at a molecular level is well established (1, 11–13). Type II TA systems constitute the largest family of TA systems and are comprised of a two-gene operon that encodes a labile protein antitoxin and a stable protein toxin. Under normal conditions, the TA forms a stable complex, which inhibits toxin activity. However, under conditions of stress, such as nutrient starvation or oxidative stress, antitoxins are rapidly degraded by cellular proteases, especially Lon and ClpXP (10, 14–20). This results in an increase of the TA ratio in the cell, eventually resulting in free toxin, which can then act on its substrates and arrest cell growth, for example, by cleaving mRNA (many toxins are RNases (1)). Despite these advances, a molecular understanding of how the likely highly specific cellular stressors target distinct antitoxins for degradation remains an open question.

MqsRA is an E. coli TA system that can be activated by oxidative stress (21). The mqsR gene encodes a 98-amino-acid sequence–specific mRNA endoribonuclease from the RelE family of bacterial toxins (Fig. 1, A and B) (11, 22, 23). Immediately downstream of mqsR is mqsA, which encodes the 131-amino-acid cognate antitoxin of MqsR and also binds the mqsRA operon to repress its transcription. MqsA binds and neutralizes MqsR toxicity via its N-terminal domain (Fig. 1, A and B) (11, 24). The selective degradation of MqsA in response to stress by the major bacterial proteases Lon and ClpXP (10, 21) results in excess MqsR toxin, which is then free to degrade the cellular mRNA pool at GCU/A sequences, and, in turn, halt translation and induce growth arrest (11, 22). However, unlike most antitoxins, MqsA is not an intrinsically disordered protein (IDP) in the absence of its cognate toxin, a structural feature hypothesized to contribute...
Regulated MqsA degradation by ClpXP

Figure 1. ClpXN and MqsR. A, MqsR and MqsA constructs used in this study. The MqsR toxin is a single domain ribonuclease. The MqsA antitoxin is a two-domain protein (MqsAN and MqsAC). MqsR binds MqsAN, and this is the domain that also binds zinc via four cysteine residues (Cys33, Cys36, Cys37, and Cys40). The C-terminal dimerization domain binds DNA; B, model of the MqsA (grays, blues; Protein Data Bank [PDB]: 3GN5) bound to MqsR (coral; PDB: 3H1Z); C, model of the MqsA dimer, with the coordinating cysteine residues shown as sticks in orange; D, ClpX N-terminal zinc-binding domain (ClpXN) is a dimer (one monomer, dark green surface; second monomer, light green ribbon; PDB: 2D56). Zinc-binding cysteine residues (Cys15, Cys18, Cys37, and Cys40) are shown as sticks in orange, with the zinc ion in teal.

Results

Zinc and MqsR protect MqsA from degradation by ClpXP

Using purified E. coli ClpX and ClpP, we reconstituted active proteasomes in vitro to monitor degradation of MqsRA proteins. We purified MqsA, with and without a bound zinc (Fig. 1, A, B, and D). MqsAF (full-length) and MqsAFL (metal-free) were incubated individually and together. ClpXP and ATP were then added, and the reactions incubated for 1 h. MqsA degradation was monitored over time by SDS-PAGE (Fig. 2A). We observed that MqsAFL is rapidly degraded, with most of the protein proteolyzed after 20 min; however, under the same conditions, MqsAF is not degraded. This suggests that zinc coordination stabilizes MqsA against ClpXP degradation.

ClpXP targeting is mediated by the MqsA N-terminal domain

The ability of zinc and MqsR to protect MqsA against ClpXP-mediated degradation suggests that ClpXP engages MqsA via its N-terminal zinc-binding and MqsR-binding domain. To test this, the N-terminal and C-terminal domains of MqsA (MqsAFL [residues 1–76]; MqsAC [residues 62–131]; Fig. 1, A and B) were prepared and tested for their ability to be degraded by ClpXP. The data show that only MqsAFL is degraded by ClpXP; MqsAC is not (Fig. 2C).
Similar to MqsA FL (Fig. 2, A and B), the majority of MqsAN is degraded in approximately 20 min. This highlights that MqsA residues required for targeting MqsA to ClpX are located within the first 76 residues of MqsA (Fig. 2C).

**Metal-loaded MqsAN does not bind ClpXN**

The N-domain of ClpX interacts directly with some substrates, such as UmuD', Lambda O protein, MuA and FtsZ, and substrate-specific adaptor proteins, such as SspB (27–32). Here, we observed that the ClpX N-domain (ClpXN; amino acids 1–55) forms a homodimer that, like MqsAN, binds zinc via four cysteines (Cys15, Cys18, Cys37, and Cys40; Fig. 1C), which is consistent with previous reports (27, 33). In order to determine how MqsAN and ClpXN interact at a molecular level, we used NMR CSP mapping, in which the changes in the chemical shifts of 15N-labeled protein are used to identify the residues that constitute a ligand-binding and/or protein-binding site. We first tested the importance of zinc for ClpXN structure by comparing the 2D [1H, 15N] heteronuclear single quantum coherence (HSQC) spectra of ClpXN in the absence and presence of zinc. The spectrum quality improves substantially in the presence of excess zinc, showing that zinc is necessary for ClpXN folding and stability (Fig. S1B); thus, zinc was included in all subsequent ClpXN experiments. CSP mapping was then used to determine if the MqsAN interacts with ClpXN. No CSPs were observed in 15N-labeled ClpXN in the presence of 10-fold molar excess of MqsAN (Fig. 3A).

These data demonstrate that metal-loaded MqsAN does not bind ClpXN, an observation consistent with the data that show that metal-loaded MqsA is resistant to ClpXP-mediated degradation (Fig. 2A).

**Figure 2. The degradation of MqsA by ClpX is inhibited by MqsR and zinc.** A, degradation reactions containing ClpX (1 μM), ClpP (1 μM), ATP (5 mM), and an ATP-regenerating system with MqsAFL (6 μM) or MqsAFL (6 μM), where indicated. Samples were taken at 0, 10, 20, 30, and 60 min and analyzed by SDS-PAGE and Coomassie staining. B, time course of MqsANL degradation in the presence and absence of MqsR (4 μM; added prior to ClpXP) with ClpX (0.5 μM), ClpP (0.6 μM), ATP, and an ATP-regenerating system. Samples were taken at 0, 5, 15, 30, and 60 min and analyzed by SDS-PAGE and Coomassie staining. C, time course of MqsANL (6 μM) (open circles) and MqsAC (6 μM) (closed circles) degradation with ClpX (1 μM), ClpP (1 μM), ATP, and an ATP-regenerating system. Band intensity was measured by densitometry, and error is reported as a standard deviation. A–C, reactions shown are representative of at least three independent replicates. MqsAC, C-terminal domain of MqsA; MqsAN, N-terminal domain of MqsA.

**Figure 3. ClpXN only binds MqsAN when it is unfolded and does not coordinate zinc.** A, overlay of 2D [15N, 1H] HSQC spectra of 15N-labeled ClpXN in the presence of increasing concentrations of MqsAN. CSPs versus residue numbers are shown below. No significant CSPs are observed. B, overlay of 2D [15N, 1H] spectra of 15N-labeled ClpXN in the presence of increasing concentrations of MqsAN, which cannot bind zinc and is unfolded. Multiple CSPs are observed. CSPs versus residue numbers are shown below; 1.5σ level is indicated by a dashed line. C, residues that experience significant CSPs are plotted on the surface of the ClpXN crystal structure (PDB: 2DS6) and highlighted in pink (≥1σ), magenta (≥1.5σ), and purple (≥2.0σ) (* indicates residues from chain B of the dimer). CSP, chemical shift perturbation; HSQC, heteronuclear single quantum coherence; MqsAN, N-terminal domain of MqsA.
Regulated MqsA degradation by ClpXP

Unfolded and metal-free MqsA binds ClpXN

Because the $K_D$ of MqsAN for zinc is estimated to be $<10^{-15} \text{M}$ (34), the zinc present in the ClpXN buffer ensures that MqsA is fully metal loaded, even if initially prepared as metal free (Fig. 1D). Thus, in order to study a WT variant that was unable to bind zinc, we generated an MqsAS deletion that lacks two of the four Cys residues required for zinc binding (Fig. 1A; residues 1–34, MqsA1–34 includes only Cys3 and Cys6). The 2D [H, 15N] HSQC of MqsA1–34 confirmed that MqsA1–34 is unfolded independent of the presence of zinc (Fig. S2A; compare also with the 2D [H, 15N] HSQC spectrum of folded MqsAN; Fig. S2B). We then completed the sequence-specific backbone assignment of ClpXN in order to probe the interaction of MqsA1–34 with 15N-labeled ClpXN at four ClpXNC:MqsA1–34 ratios (1:1; 1:2; 1:4; and 1:8). Upon the addition of MqsA1–34, 17 CSPs were observed in 15N-labeled ClpXN (<1.5σ), with the strongest CSPs observed for residues Asp3, Lys4, Leu13, Lys27, Ile29, and Ala30 (Fig. 3, B and C). To confirm that the interaction is specific for MqsA1–34 and not because it is an IDP, we performed CSP experiments with two distinct and well-characterized IDPs: PNUTS376–425 or inhibitor-2 (Fig. S3). No significant CSPs in 15N-labeled ClpXN were observed when titrated with either PNUTS376–435 and Inhibitor-210–165, demonstrating that the MqsA interaction is specific. Together, these data show that ClpXN binds directly and specifically to residues within the first 34 amino acids of MqsA.

Identification of the MqsAN motif recognized by ClpX

To identify the MqsA residues that bind ClpXN, we performed reverse CSP experiments. After completing the sequence-specific backbone assignment of MqsA1–34, increasing concentrations of ClpXN were added to 15N-labeled MqsA1–34, and the resulting CSPs identified. Seven residues experienced significant CSPs (>1.5σ): Ile18, Thr21, Phe22, Gly24, Leu29, Ile32, and Gly34, with one peak, Gly31, broadened beyond detection (Fig. 4). Most of these residues are buried in the hydrophobic core of folded MqsA, with Phe22, Leu29, and Ile32 being nearly completely buried from solvent (percent of solvent accessibility in folded MqsA: 20.8, 3.6, and 4.1, respectively). These data identify the MqsA residues that mediate ClpXN recognition and explain why folded MqsAN, in which these residues are buried in the hydrophobic core, are unable to bind ClpXN.

MqsA1–34 contains a transplantable degron, the MqsA degron, for ClpXP

To determine if an MqsA1–34 peptide sequence is capable of independently targeting a protein for degradation, we produced a chimeric fusion protein with Gfp fused to the N-terminus of MqsA1–34, and measured degradation by monitoring loss of fluorescence after incubation with ClpXP in vitro. Gfp, without a specific ClpX degron, is not recognized or degraded by ClpXP (35); however, if MqsA1–34 is recognized by ClpX, then we predict that ClpXP will unfold and degrade Gfp-MqsA1–34, leading to a loss of Gfp fluorescence. In the absence of ClpXP, Gfp-MqsA1–34 fluorescence is stable over time (Fig. 5A). In the presence of ClpXP, we observed a rapid loss of fluorescence, demonstrating that ClpX binds MqsA1–34 resulting in Gfp-MqsA1–34 unfolding and subsequent degradation by bound ClpP. Consistent with these data, we also showed that the turnover of Gfp-MqsA1–34 is diminished in the ClpXP protease deletion strains (Fig. 5B). These data confirm that MqsA contains a cryptic N-domain recognition sequence, the MqsA degron, that is accessible only in the absence of zinc and MqsR toxin and that this recognition sequence is transplantable and sufficient to target a fusion protein (Gfp-MqsA1–34) for degradation in vitro and in vivo.

We then confirmed the identity of the residues that define the MqsA1–34 binding pocket on ClpXN using mutagenesis and fluorescence degradation assays. Specifically, we mutated ClpXN residues that (1) experienced CSPs upon titration with MqsA1–34; (2) are solvent accessible; and (3) are unlikely to disrupt nearby regions upon mutagenesis (L12, L13, H23, and A30) (Fig. 3, B and C). We first confirmed that the engineered ClpX variants (ClpX[L13A], ClpX[L13D], ClpX[A30S], ClpX[L12S], and ClpX[H23A]) were properly folded and functional by showing (1) that the ClpXN variants were folded using 1H 1D NMR spectroscopy; (2) that all full-length ClpX variants, like WT ClpX, purified as assembled hexamers by size-exclusion chromatography (SEC); (3) that the full-length ClpX variants hydrolyzed ATP; and (4) that the full-length ClpX variants complex with ClpP to degrade Gfp-ssrA, which bypasses the ClpX N-domain (Fig. S4). Next, ClpX variants were tested in degradation assays with ClpP and Gfp-MqsA1–34. The data show that degradation for ClpX variant A30S was similar to WT ClpX, but degradation was...
significantly reduced for ClpX variants, L12S, L13A, L13D, and H23A (Fig. 5A). To confirm that the slower degradation rates observed were due to a reduction in MqsA1–34 binding to ClpX, and not because of a defect in delivery or translocation through the ClpX pore, we used ClpXP filter retention assays. Consistent with the degradation results from ClpX variants, we observed that Gfp-MqsA1–34 was retained by WT ClpX and ClpX A30S, but retention was defective for ClpX variants L12S, L13A, L13D, and H23A (Fig. 5C). Together, these results confirm that the region of the ClpX N-domain implicated in binding to MqsA by NMR is important for recognition and degradation by ClpXP.

The MqsA1–34 binding pocket on ClpXN overlaps, but is not identical, to that of SspB

The amino acid residues in the ClpX N-domain implicated in MqsA recognition are close to or overlapping with residues, including H23, identified as important for binding to the C-terminus of the SspB adaptor protein. To determine if the SspB adaptor protein and MqsA use overlapping sites on the surface of ClpX, we tested if a peptide containing 11 C-terminal amino acid residues of SspB inhibits degradation of MqsA_{nf} a similar peptide was previously shown to inhibit degradation of ssrA-tagged substrates in SspB adaptor-mediated degradation reactions (36). The data show that the C-terminal SspB peptide reduced MqsA_{nf} degradation, confirming that the binding sites for SspB and MqsA on ClpXN overlap (Fig. 5D).

Discussion

Stress accelerates antitoxin turnover via regulated proteolysis (10, 14, 15, 20); however, a molecular understanding of how antitoxins are targeted for degradation by Lon and/or ClpXP remains an open question (10, 17–20, 37). Here, we show how the MqsA antitoxin, because of the presence of a novel cryptic MqsA degron that is only accessible in unfolded MqsA, is recognized and degraded by ClpXP. Specifically, we discovered, unexpectedly, that folded MqsA does not interact with ClpXN, even with weak affinities. Consistent with this, folded MqsA is not degraded by ClpXP. Instead, we showed that ClpXN only binds MqsA when MqsA is unfolded. This led to the discovery of a cryptic ClpXN recognition motif...
within the first 34 residues of MqsA that is buried when MqsA is folded but becomes accessible when MqsA is prevented from folding. Furthermore, we observed that the chimeric Gfp-MqsA1–34 fusion protein, in which the MqsA remains flexible, is degraded by ClpXP in vitro and in vivo (Fig. 5B). Thus, the ClpX recognition motif of MqsA is transplantable, defining a novel ClpX degron, the MqsA degron, with an amino acid sequence that differs from all previously described degrons (38).

Because the MqsA degron is only accessible in unfolded MqsA, interactions that stabilize folded MqsA, such as zinc and/or MqsR binding, potently inhibit ClpXP-mediated MqsA degradation. These in vitro data are fully consistent with recent in vivo experiments showing that, in cells, both MqsA and a second antitoxin, YefM, are protected from degradation when bound to their cognate toxins (10, 20). By extension from our data, it is likely that the YefM toxin-binding domain also contains a degron that is accessible only in the absence of toxin. According to these structurally dependent events, this leads to the following question: under what conditions are MqsA (unfolded and cryptic ClpXN degron accessible) and YefM (not bound to toxin) susceptible to degradation? In the case of MqsA, actively translated and nascent MqsA is the likely target. This is because, during synthesis and prior to folding, the cryptic MqsA degron would be accessible. Alternatively, in oxidative conditions, the cysteines of nascent MqsA could be oxidized prior to metal binding, rendering them unable to bind zinc and fold, also permanently exposing the MqsA degron following synthesis (Fig. 6). Both scenarios would render the MqsA degron accessible for binding. Consistent with this possibility, MqsA protein has been shown, in vivo, to be degraded under oxidative conditions (10, 21) (exposure to H₂O₂, a chemical that readily oxidizes cysteines). Because H₂O₂ fails to oxidize metal-loaded MqsA (the zinc-coordinating cysteine residues are protected from oxidation by the bound zinc), this leads to a model in which only actively translated and nascent MqsA is the predominant target. Similarly, synthesized toxin-free YefM or nascent YefM is the only YefM species targeted for degradation.

Whether this results in the activation of MqsR depends on the extent of the exposure to the stress. Prior to recent work, including this study, the prevailing model for toxin activation had been that stress results in the selective degradation of antitoxins within a TA complex, “freeing” the toxin to exert its toxic activities and arrest cell growth (1, 4). However, our data, and that of others, have shown this model is incorrect as toxins bound to their cognate antitoxins clearly inhibit antitoxin degradation (10). Thus, while stress results in the selective degradation of free and nascent and/or unfolded antitoxins, it does not result in the degradation of antitoxins present in TA complexes. Therefore, the antitoxin-bound toxins will never become “free” to exert their toxic effects. However, despite this discovery, this does not mean toxins are never active. Namely, when toxin concentrations exceed those of their cognate antitoxins, the excess cellular toxins will be free to act on their endogenous substrates, and, in turn, inhibit cell growth.

Figure 6. Model of MqsA degradation by ClpXP and activation of the MqsR toxin. Under normal conditions (top), both MqsA and MqsR are translated and folded, leading to complex formation and toxin inhibition. Under stress conditions, MqsR folds, whereas MqsA does not (MqsAu), for example, because of oxidation of the four cysteine residues that coordinate zinc. This exposes a cryptic ClpXN-binding motif on MqsA, resulting in ClpXN binding and ClpXP-mediated degradation. MqsR is then free to cleave its mRNA substrates, ultimately resulting in growth arrest.
Antitoxin degradation potently activates TA operon transcription. In most cases, this results in sufficient expression of antitoxin to match or exceed the concentration of toxin (Fig. 6). However, it is possible that extended exposure to stress may cause this balance to shift, resulting in excess toxin and growth arrest (Fig. 6). For example, although it is established that MqsA transcription and expression exceeds that of MqsR, if all newly synthesized MqsA is degraded, eventually the newly synthesized MqsR will not have a cognate antitoxin available for binding, allowing it to target and cleave its endogenous substrate GCU/A mRNA sequences. The endogenous environmental stressors (and the extent to which they must be exposed) that ultimately activate MqsR via MqsA degradation, and other TA systems, as well as regulated proteolysis by other proteases, such as Lon, are still active areas of investigation.

**Experimental procedures**

**Cloning, expression, and purification**

MqsAN and ClpXN were subcloned in the pRIB vector, containing a cleavable N-terminal hexahistidine (His6) tag followed by a tobacco etch virus (TEV) cleavage sequence. To facilitate stable expression, MqsA1 was subcloned into the pTHMT vector, which includes an N-terminal maltose-binding protein followed by a TEV cleavage sequence. Mutagenesis was carried out using the QuickChange Mutagenesis Kit (Agilent Technologies) using the manufacturer’s protocols; all constructs were verified by sequencing. For expression, plasmid DNAs were transformed into E. coli BL21(DE3) cells. Cells were grown with shaking at 37 °C in either LB for nonlabeled proteins or M9 minimal media containing 1 g/l of 15NH4Cl and 4 g/l of d-glucose as the sole nitrogen and carbon sources, respectively, with appropriate antibiotics. Once the cultures reached an absorbance of ~0.8 at 600 nm, they were cooled at 4 °C for 1 h, 1 mM IPTG was added to induce expression, and the cultures were transferred to 18 °C for 10 h with shaking. Cells were harvested by centrifugation at 8000 g for 10 min, and pellets were stored at −80 °C.

MqsAN was purified as previously described (11). Briefly, cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100, and EDTA-free protease inhibitor), lysed by high-pressure cell homogenization (Avestin C3-EmulsiFlex), and the cell debris was pelleted by centrifugation (42,000g, 45 min). The supernatant was filtered with 0.22 μm syringe filters, loaded onto a HisTrap HP column pre-equilibrated with buffer A (50 mM Tris, pH 8.0, 500 mM NaCl, and 5 mM imidazole), and eluted using a linear gradient of buffer B (50 mM Tris, pH 8.0, 500 mM NaCl, and 500 mM imidazole). Fractions containing MqsAN were pooled and dialyzed overnight at 4 °C with TEV protease to cleave the His6 tag. The cleaved protein was incubated with Ni2+-NTA resin (GE Healthcare), and the flow-through was collected. The protein was concentrated and purified using SEC (Superdex 75 26/60 [GE Healthcare]) pre-equilibrated with NMR buffer 1 (20 mM Na/PO4, pH 6.5, 50 mM NaCl, and 0.5 mM Tris(2-carboxyethyl)phosphine [TCEP]). ClpXN and MqsA1−34 were purified similarly but with the following differences. The ClpXN sizing buffer included 0.5 mM ZnSO4 (20 mM Na/PO4, pH 6.5, 50 mM NaCl, 0.5 mM TCEP, and 0.5 mM ZnSO4). Prior to SEC, the MqsA1−34 was heat purified twice by incubating the sample at 80 °C for 10 min. The sample was then centrifuged at 15,000g for 10 min to remove precipitated protein, concentrated, and purified using SEC (Superdex 75 26/60) in NMR buffer.

MqsR was also purified as described previously (39). Briefly free and folded MqsR is obtained by coexpressing pET30a-MqsA (which includes an N-terminal His6 tag), with untagged pCA21a-MqsAN 144A (MqsAN is the N-terminal domain of MqsA and includes residues 1–76) at 18 °C in BL21(DE3) E. coli competent cells. After lysis, the His6-MqsR–MqsAN complex was bound to nickel metal affinity resin and denatured with 6 M guanidine hydrochloride. His6-MqsR (hereafter referred to as MqsR) was eluted with 500 mM imidazole and refolded by stepwise dialysis in buffers containing decreasing amounts of guanidine hydrochloride followed by a final preparative gel filtration step (Superdex 75 16/60; GE Healthcare; SEC buffer, 10 mM Tris, pH 7.5, 50 or 100 mM NaCl, and 0.5 mM TCEP).

To construct Gfp-MqsA1−34, genes encoding Gfp and MqsA were cloned into pBad24 (40), a stop codon was introduced by site-directed mutagenesis at codon 35 of mqsA, and mutagenesis was confirmed by direct sequencing. Gfp-MqsA1−34 expression was induced in log phase E. coli BL21(ADE3) ΔclpP::cat cells with arabinose (0.2%) for 6 h by shaking at 25 °C. Cells were harvested, lysed by French press, and Gfp-MqsA1−34 was purified from soluble cell lysates by organic extraction and phenyl Sepharose chromatography as described (41). Gfp-ssrA, ClpX, and ClpP were overexpressed in E. coli BL21(ADE3) and purified as previously described (32, 41–43). ClpX N-domain mutations were introduced into pET-ClpX by site-directed mutagenesis, confirmed by direct sequencing, and purified as WT ClpX. ClpX WT and N-domain variants were fractionated on Sephacyrl S-200 HR (GE Healthcare), and hexamers were collected. To confirm enzymatic activity, ATP hydrolysis was monitored by measuring phosphate release with Biomol green reagent (Enzo Life Sciences) in reactions containing ClpX WT and mutant proteins (0.5 μM) and ATP (5 mM) in 50 mM Heps, pH 7.5, 150 mM KCl, and 20 mM MgCl2, at 23 °C. A-terminal SspB peptide (NH2-RGGRPALRVVK-COOH) was synthesized and purchased from Life Technologies. Protein concentrations are reported as Gfp monomers, ClpX hexamers, and ClpP tetradecamers.

**Preparation of metal-free MqsA for degradation experiments**

Purified MqsA constructs were diluted to 10 μM in the presence of 10 mM EDTA, heated for 10 min at 80 °C, and then allowed to cool to room temperature for 5 min. Samples were then dialyzed against 10 mM Tris–HCl, pH 7.0, 50 mM NaCl, and 0.5 mM TCEP overnight at 4 °C. Prior to freezing, the samples were centrifuged at 15,000g for 10 min at 4 °C to remove any precipitated protein.
Regulated MqsA degradation by ClpXP

Sequence-specific backbone assignment

\[ ^{15}\text{N}, ^{13}\text{C}-\text{labeled MqsA}_{1-34} (85 \mu\text{M}; \text{concentrations greater than 100 \muM precipitated}) \text{ and } ^{15}\text{N}, ^{13}\text{C}-\text{labeled MqsA}_{N} (650 \mu\text{M}) \text{ were prepared in NMR buffer 1, whereas } ^{15}\text{N}, ^{13}\text{C}-\text{labeled ClpX}_{N} (800 \mu\text{M}) \text{ was prepared in NMR buffer containing 0.5 mM ZnSO}_4; 10\% (v/v) \text{ D}_2\text{O was added immediately prior to data acquisition. The sequence-specific backbone assignments of ClpX}_{N}, MqsA_{1-34}, \text{ and MqsA}_{N} \text{ were determined by recording a suite of heteronuclear NMR spectra including: 2D \[ {^1\text{H}, ^{15}\text{N}}\] HSQC, 3D HNCA, 3D HN(CO)CA, 3D HNCACB, and 3D CBCA(CO)NH. For ClpX}_{N}, the sequence-specific backbone assignments were verified and expanded using a 3D HNCO, 3D HN(CA)CO, and 3D (H) CC(CO)NH. All NMR data were collected on a Bruker Advance Neo 600 MHz Spectrometer and an 800 MHz Spectrometer equipped with TCI HCN z-gradient cryoprobe at 298 K. Data were processed using Topspin (Bruker) and analyzed using CARA (http://www.cara.nmr.ch) and CCPN (44, 45). To test the role of zinc for ClpX}_{N} sequence quality, \text{ZnSO}_4 (100 \mu\text{M}) was titrated into 15N-labeled ClpX}_{N} (200 \mu\text{M}) was purified as described with the exception that the NMR buffer did not contain ZnSO}_4. After recording a 2D \[ {^1\text{H}, ^{15}\text{N}}\] HSQC spectrum, 500 \mu\text{M} ZnSO}_4 was added, and a 2D \[ {^1\text{H}, ^{15}\text{N}}\] HSQC spectrum was recorded and a direct comparison performed.

MqsA-ClpX}_{N} NMR spectroscopy interaction studies

All NMR data were collected on a Bruker Advance Neo 600 MHz Spectrometer equipped with TCI HCN z-gradient cryoprobe at 298 K. MqsA_{1-34}, MqsA}_{N}, and ClpX}_{N} were purified in NMR buffer containing 0.5 mM ZnSO}_4. MqsA}_{N} was titrated into 15N-labeled ClpX}_{N} (50 \mu\text{M}) in molar ratios of 1:1, 1:2, 1:5, and 1:10 and 2D \[ {^1\text{H}, ^{15}\text{N}}\] HSQC spectra were recorded, resulting in no observable interaction. MqsA}_{1-34} was titrated into 15N-labeled ClpX}_{N} (50 \mu\text{M}) in ratios of 1:1, 1:2, 1:4, and 1:8, with 2D \[ {^1\text{H}, ^{15}\text{N}}\] HSQC spectra recorded for each sample. These titrations were used to follow CSPs in ClpX}_{N} upon binding; the majority of peaks are in the fast exchange regime and thus can be readily traced. ClpX}_{N} was titrated into 15N-labeled MqsA}_{1-34} in ratios of 1:0.5, 1:1, 1:2, and 1:4 with 2D \[ {^1\text{H}, ^{15}\text{N}}\] HSQC spectra recorded for each sample. These titrations were used to follow CSPs in MqsA}_{1-34} all peaks are in the fast exchange regime and thus can be readily traced. Chemical shift differences (Δδ) were calculated using the following equation:

\[
\Delta \delta(p.p.m.) = \sqrt{(\Delta \delta_H)^2 + \left(\frac{\Delta \delta_N}{5}\right)^2}
\]

Proteolysis and direct binding assays

MqsA}_{FL}, MqsA}_{FLm}, MqsA}_{NP}, or MqsA}_{NL} (6 \mu\text{M}) was incubated with ClpX (1 \mu\text{M}) and ClpP (1 \mu\text{M}), or as indicated, in 50 mM Mes buffer, pH 6.5, 110 mM KCl, 20 mM MgCl}_2, 5 mM ATP, and acetate kinase (25 \mu\text{g ml}^{-1}) (Sigma–Aldrich) and 15 mM acetyl phosphate (Sigma–Aldrich) at 23 \text{°C}. At the indicated times, samples were removed, and reactions were analyzed by SDS-PAGE and Coomassie staining. Where indicated, purified MqsR (10 \mu\text{M}) and SspB peptide (150 \mu\text{M}) were included in the degradation reactions. To monitor degradation by loss of fluorescence, Gfp-MqsA}_{1-34} (0.5 \mu\text{M}) was incubated with ClpX (0.75 \mu\text{M}) and ClpP (1.2 \mu\text{M}) in degradation buffer (50 mM Hepes, pH 7.0, 100 mM KCl, 20 mM MgCl}_2, 0.005% Triton X-100, 5 mM ATP, 25 \mu\text{g ml}^{-1} \text{acetic kinase, and } 15 \mu\text{M acetyl phosphate}) at 23 °C. Fluorescence was monitored using an Agilent Eclipse spectrofluorometer at excitation and emission wavelengths of 420 and 510 nm, respectively.

To detect substrate binding, ClpX WT and N-domain variants (0.7 \mu\text{M}) were incubated with ClpP (1.4 \mu\text{M}) and Gfp-MqsA}_{1-34} (3 \mu\text{M}) in substrate binding buffer (50 mM Hepes, pH 7.0, 100 mM KCl, 10 mM MgCl}_2, 0.004% Triton X-100, 50 \mu\text{g ml}^{-1} \text{bovine serum albumin, and } 5 \mu\text{M ATP}) on ice for 15 min. Stable complexes were collected by centrifugation at 20,000 g for 20 min and retained on a Nanosep polyethersulfone filter ( Pall) with a molecular weight cutoff of 100 kDa. Retained complexes were collected, and substrate was quantified by fluorescence.

Antibiotic chase assays

The pBAD24 vector encoding Gfp-MqsA}_{1-34} was transformed into MG1655 WT and MG1655 ΔclpPΔ::kan strains. Overnight cultures were grown and back-diluted the following day to an absorbance of 0.05 at 600 nm in a 30 ml culture. Ampicillin (100 \mu\text{g ml}^{-1}) and l-arabinose (0.01%) were added, and cultures were grown at 37 °C until reaching an absorbance of 0.7 at 600 nm. Spectinomycin (200 \mu\text{g ml}^{-1}) was added to the cultures to stop protein synthesis, and cells were collected at 0, 5, 10, 15, and 30 min. Total cellular proteins were immediately precipitated with trichloroacetic acid (Sigma) (15% v/v) as described (32). Protein turnover was monitored by Western blot using anti-Gfp rabbit IgG (Invitrogen), Horseradish peroxidase–linked anti-rabbit IgG (Cell Signaling Technology) and chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific). Band intensity was quantified by densitometry (Image), the National Institutes of Health)

Data availability

NMR chemical shifts have been deposited in the Biological Magnetic Resonance Data Bank (BioMagResBank or BMRB: 50781, 50782, and 50784).

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CSP, chemical shift perturbation; Gfp-ssrA, Gfp fused to the ssrA tag sequence; His6, hexahistidine tag; HSQC, heteronuclear single quantum coherence; IDP, intrinsically disordered protein; MqsA_C, C-terminal domain of MqsA; MqsA_N, N-terminal domain of MqsA; MqsAFL, full-length MqsA; SEC, size-exclusion chromatography; TA, toxin–antitoxin; TCEP, Tris(2-carboxyethyl)phosphine; TEV, tobacco etch virus.

References
Regulated MqsA degradation by ClpXP


