Periplasmic Chaperones and Prolyl Isomerases

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ABSTRACT The biogenesis of periplasmic and outer membrane proteins (OMPs) in *Escherichia coli* is assisted by a variety of processes that help with their folding and transport to their final destination in the cellular envelope. Chaperones are macromolecules, usually proteins, that facilitate the folding of proteins or prevent their aggregation without becoming part of the protein's final structure. Because chaperones often bind to folding intermediates, they often (but not always) act to slow protein folding. Protein folding catalysts, on the other hand, act to accelerate specific steps in the protein folding pathway, including disulfide bond formation and peptidyl prolyl isomerization. This review is primarily concerned with *E. coli* and *Salmonella* periplasmic and cellular envelope chaperones; it also discusses periplasmic proline isomerization.

BIOGENESIS OF EXTRACYTOPLASMIC PROTEINS

In Gram-negative bacteria, proteins destined for the periplasm or outer membrane are, in the vast majority of cases, synthesized in the cytoplasm as precursors with a cleavable N-terminal signal sequence and then exported to their final destination in the cell envelope. Although various pathways exist for their transport across the inner membrane, most precursor proteins are translocated in unstructured states through an inner membrane channel formed by the heterotrimeric membrane complex SecYEG ([1]). After translocation and signal sequence cleavage, the newly exported extracytoplasmic proteins are sorted inside the periplasm via different folding pathways. While periplasmic localization is generally thought to be the default destination for proteins carrying cleavable signal sequences ([2]), folding intermediates of outer membrane proteins must transit through the periplasm in a conformational state compatible with their insertion into the lipid bilayer ([3]). Lipoproteins have their own dedicated secretion mechanism and chaperone, LolA. Lipoprotein secretion has recently been reviewed ([4]); it is not further described here.

The periplasm is separated from the extracellular milieu by only the porous outer membrane, which allows entry of anything below ∼600 Da, and is thus more susceptible to changes in the external environment than the cytoplasm. Periplasmic proteins must be able to cope with extreme changes in the

environment. It is therefore not surprising that soluble periplasmic proteins are often highly stable, making them resistant to unfolding and aggregation (5). A disproportionate percentage of periplasmic proteins have had their structures determined by X-ray crystallography, which is understandable since highly stable proteins are often easier to crystallize (6). Given the high stability of periplasmic proteins, until fairly recently it was not even clear whether general periplasmic chaperones exist (7). However, as described below, it is now evident that there is an abundance of periplasmic chaperones, whose activity is particularly important under stress conditions.

The protein folding environment of the periplasm presents some unique differences from the protein folding challenges encountered in the cytosol. For example, the periplasm lacks ATP. Thus, it is clear that periplasmic chaperones must act using at least somewhat different mechanisms than those employed by cytosolic ATP-dependent chaperones. Another difference is that the cytosol is a reducing environment, whereas the periplasm is oxidizing. Disulfide bond formation and isomerization in periplasmic proteins is driven by the disulfide bond (DSB) family of enzymes. Null mutants in these DSB catalysts lead to steep declines in the abundance of a wide variety of proteins that contain disulfides necessary for their folding or stability (8, 9). The disulfide bond catalysts are not further discussed here, except to mention that DsbC and DsbG have both been shown to have chaperone activity *in vitro* (10, 11).

Proteins destined for the outer membrane or the exterior of the cell, such as pili, are not folded in the periplasm. They are instead kept in a partially unfolded, secretion-competent conformation as they transit the periplasm. The driving force for this transport and secretion may simply be the thermodynamic sink provided by outer membrane protein insertion into the outer membrane (12).

STRESS CHAPERONES

Although relatively few chaperones are involved in the folding of soluble periplasmic proteins under laboratory conditions, in nature, the *E. coli* periplasm is commonly faced with crises that can tax even the most stable proteins. Given the permeability of the outer membrane, the periplasm is especially vulnerable to the variety of different chemical stresses that Gram-negative bacteria are exposed to. These chemical agents can induce un-

folding and/or aggregation of the periplasmic proteome. Thus far, several chaperones have been identified that combat periplasmic protein unfolding due to chemical agents. During transit through the stomach, for example, the *E. coli* periplasm is exposed to acid, which is a potent denaturant, or if swallowed by a herbivore, can be exposed to tannins, which are common in plants and are potent protein aggregation agents (13). As discussed below, periplasmic proteins require so much assistance remaining soluble under these conditions that *E. coli* induces chaperones to levels that approach 50% of the periplasmic proteome.

In addition to being required during stress and for outer membrane protein insertion, there are a number of elongated specialized structures present on the surface of bacteria, including pili, fimbriae, and curli, that require specialized chaperones for their assembly. Many of these structures appear to operate via a β-strand displacement mechanism, which we discuss further below.

REGULATORY MECHANISMS FOR STRESS-SPECIFIC PERIPLASMIC CHAPERONES

Organisms have evolved so that the expression level of periplasmic chaperones adapts to meet demand. The Cpx, sigmaE, Bae, Psp, and Rcs pathways in *E. coli* all sense alterations in the bacterial envelope and respond by inducing a variety of components involved in the formation and maintenance of the bacterial envelope, including many periplasmic chaperones and folding factors (14). The protease/chaperone DegP and the prolyl isomerase FkpA, for example, are induced by both the Cpx and sigmaE pathways. The chaperone Spy is induced by the Bae, Cpx, and RcsB pathways, whereas the outer membrane insertion chaperone SurA is only induced by the sigmaE pathway. These pathways are activated by a variety of stressors (15). For example, the potent protein denaturant, ethanol, activates all five pathways, suggesting that all of these pathways are responsive to protein unfolding (16). However, tannins only induce the Bae pathway. Bae induction by tannins is so strong that the Bae-controlled chaperone Spy becomes nearly half of the periplasmic mass after tannin treatment (13, 16). Acid treatment is also known to unfold proteins while also strongly inducing expression of the acid-responsive chaperones HdeA and HdeB (17).

Different protein denaturants act with varying degrees of effectiveness on different sets of proteins and induce

distinct unfolded state ensembles ([18](18)). Thus, it is not surprising that different chaperones are needed to cope with these various denatured states. It seems likely that different chaperones have evolved to recognize not just different proteins but also different unfolded states of the same protein. Characterizing the presumably complex and overlapping substrate specificity of the various periplasmic chaperones is in its early stages.

The Cpx and Bae responses are classic two-component regulatory systems ([19](19), [20](20)). The Cpx response is one means of detecting and responding to periplasmic protein folding problems ([21](21)). Many of the Cpx inducers, including overexpression of misfolded proteins, disruption of periplasmic disulfide bond formation, and treatment with denaturing alcohols act to disturb envelope folding. About 50 transcriptional units are induced by the Cpx response, many of which are cell envelope proteins ([14](14)). Some of the proteins most strongly induced by the Cpx response encode periplasmic folding and degradation factors, including Spy, DsbA, DegP, and HtpX. The Cpx response also plays roles in virulence, pili formation ([22](22)), and drug resistance ([23](23)), and helps regulate inner membrane transport and energy metabolism ([21](21)). The Bae response, in contrast, triggers the induction of only about 7 transcriptional units. Since Spy, a periplasmic chaperone, is by far the most strongly induced protein, it seems likely that the Bae response is mainly also designed to deal with protein folding issues. However, the Bae response also induces multidrug exporters; thus, this regulon may also help deal with toxic small molecules ([24](24)).

Both the Cpx and Bae systems consist of a histidine sensor kinase, CpxA or BaeS, respectively, that acts to autophosphorylate its own transmitter domain ([24](24)). Activation is achieved after this phosphoryl group is transferred to the receiver domain of the response regulator CpxR or BaeR, respectively. These regulators then act as transcription factors to activate the Cpx and Bae responses ([14](14)). CpxP, the protein most strongly induced by the Cpx response, acts as an auxiliary regulator by sensing misfolded periplasmic proteins and modulating the activity of CpxA, likely by downregulating its autokinase activity ([25](25)). CpxP is homologous to the periplasmic chaperone Spy and the periplasmic zinc binding proteins, ZraP and CnrX ([26](26), [27](27)). CpxP has weak chaperone activity *in vitro* ([13](13)), but this is most likely a by-product of its need to bind and sense misfolded periplasmic proteins for regulating CpxA rather than its functioning directly as a general chaperone *in vivo*. Most

chaperones are highly abundant proteins, but CpxP is only present at about 40 molecules per cell ([28](28)).

The Rcs system also acts through a phospho-relay system. For the sigmaE stress regulon, the accumulation of outer membrane porins and lipopolysaccharides act as inducing signals that cause the release of the transcription factor sigmaE through the regulated proteolysis of an inner membrane protein RseA, which normally acts to sequester sigmaE during nonstress periods. The Psp envelope stress-responsive system also acts by sequestering a transcription factor, in this case PspF, a sigma54 enhancer that is normally sequestered through binding by PspA. PspB and PspC bind to PspA after they themselves sense the activation signal, causing the release of PspA from PspF and thus activation of sigma54.

STRESS-SPECIFIC CHAPERONES

HdeA and HdeB

E. coli must transit through the acidic stomach of warm-blooded animals to colonize the intestines. Whereas the cytosol is protected from the low pH environment because of the relative impermeability of the inner membrane to protons, the periplasmic proteome is unprotected from this acid stress (pH ~2) because of the high permeability of the outer membrane to small molecules (<600 Da). Since the periplasm is devoid of ATP, the canonical ATP-dependent foldase chaperones of the cytosol are unavailable to protect periplasmic proteins from the acid stress.

However, *E. coli* contains two small (~10 kDa) chaperones called HdeA and HdeB that protect the periplasmic proteome during acute acid stress ([17](17), [29](29)). Both the *hdeA* and *hdeB* genes share an operon and are located on an acid fitness island ([30](30)). HdeA is reported to be the 6th most abundant protein in the cell during stationary phase ([31](31)). This point is made more remarkable by the fact that HdeA is localized to the periplasm, a compartment that takes up only 10 to 20% of the total cell mass. HdeA's abundance likely reflects the cell's need to be prepared for two considerations that occur during acid stress: (1) nearly all proteins unfold in response to acid stress, and (2) the decline in pH that accompanies ingestion of bacteria into the mammalian gut is so fast that it precludes effective transcriptional or translational responses.

A number of reports suggest that HdeA functions at extreme acidic pH values (pH 1–3), whereas HdeB is

active under more mild acid stress (pH 4–5) ([32](#)–[34](#)). Both proteins suppress the aggregation of proteins at their respective optimal pH values. Indeed, several *in vivo* cross-linking studies have revealed that HdeA and HdeB both bind a broad range of the periplasmic proteome, albeit at different pH conditions and with differing substrate specificity ([35](#)). Although HdeA and HdeB have only 17% sequence identity, they are structurally similar. Both proteins exist as well-folded α-helical dimers at neutral pH that bury large hydrophobic surfaces in their dimer interfaces ([36](#)). HdeB appears to be less important than HdeA for acid resistance; naturally occurring mutants that disrupt the ATG codon of HdeB even exist in some *E. coli* strains.

The chaperone activity of both HdeA and HdeB is regulated at the posttranslational level, which is likely necessary to respond to rapid changes in environmental pH. On exposure to low pH, HdeA undergoes a rapid, dramatic conformational change from a well-folded chaperone-inactive α-helical dimer ([36](#)) to partially disordered chaperone-active monomers ([37](#)–[39](#)). The large hydrophobic stretches buried in the dimer-dimer interface at neutral pH have been shown to be the substrate binding site of the HdeA monomers. Computational and mutagenesis studies revealed that the protonation of two aspartic acids in key salt bridges is necessary to monomerize HdeA ([39](#)–[43](#)). After activation, the chaperone-active monomers then tightly bind (nM K_d values) to substrate proteins unfolded by the acid stress to prevent them from aggregating. Interestingly, the HdeA monomers adopt different conformations when bound to different substrates, illustrating how HdeA can promiscuously bind to a variety of different client proteins. Return to neutral pH causes the release of bound substrates, allowing them to refold back into their native state, and HdeA returns to its chaperone-inactive dimer conformation.

In contrast, HdeB remains dimeric at its chaperone-active pH values. However, the HdeB dimer appears to undergo some structural rearrangements between pH 7 and pH 4, and the loops between helices 2 and 3 become less dynamic when HdeB is transitioned from pH 7 to pH 4 ([44](#)). The actual substrate binding site on HdeB has remained elusive.

Spy

Spy (Spheroplast Protein Y) ([45](#)) was discovered as a chaperone by a genetic selection that links protein sta-

bility to antibiotic resistance in the *E. coli* periplasm ([13](#)). The selection used a biosensor in which an unstable variant of immunity protein 7 (Im7) was inserted between two halves of β-lactamase, an enzyme that confers antibiotic resistance to penicillin. Spy was identified by its ability to enhance the stability of the Im7 insert and therefore enhance the antibiotic resistance of *E. coli* carrying the biosensor. Spy is a small (16 kDa) ATP-independent chaperone that can prevent aggregation of a variety of proteins and can promote the refolding of client proteins at substoichiometric concentrations ([13](#)). The crystal structure of Spy ([13](#), [46](#)) shows that its active form is a thin, cradle-shaped all α-helical dimer with a large hydrophobic surface in the middle of the cradle surrounded by basic residues on the edges of the cradle. It is normally not expressed under nonstress conditions but is massively upregulated upon protein unfolding stresses such as ethanol, butanol, or tannins, comprising a high percentage of the periplasmic proteome during these stress conditions ([16](#), [47](#)). Moreover, deletion of the *spy* gene makes *E. coli* more sensitive to tannin treatment. Its expression is controlled by the Cpx and Bae systems, both of which respond to protein unfolding stress in the cellular envelope. Spy mutants are sensitive to and accumulate zinc. It is not yet clear if this is due to an effect of Spy on zinc transport proteins or if Spy directly binds zinc ([48](#)). However, the Spy homologue, ZraP, is a zinc binding protein and also has chaperone activity *in vitro* ([27](#)).

The mechanism of chaperone action for Spy on its client, Im7, has been investigated in great detail ([49](#)–[51](#)). Unlike most chaperones, Spy uses complementary charge-charge interactions to bind to its unfolded substrates. Long-range electrostatic interactions between the positively charged binding site on Spy and negatively charged substrates accelerates Spy's association rate constant for substrates so that client binding is diffusion limited. This allows Spy to rapidly associate with unfolded, aggregation-prone clients to prevent their aggregation. After recognition of clients through long-range electrostatics, hydrophobic interactions between the central Spy cradle and the unfolded substrate stabilize the complex ([50](#)). Remarkably, Spy then allows Im7 to fold from its unfolded conformation into its native structure while remaining continuously bound to the chaperone surface ([51](#)). By doing so, Spy minimizes the concentration of unbound protein folding intermediates that would otherwise be aggregation prone. Upon substrate folding, the hydrophobic contacts between Spy and its client are reduced, which increases the dissociation rate constant

of the now-native client from Spy and causes release of the native client. Thus, client refolding triggers its own release from Spy, precluding the need for any cofactors or cochaperones to regulate Spy's chaperone function (50). Recent crystallographic experiments using an iodine-labeled Im7 substrate have captured snapshots of Im7 as it refolds while continuously bound to the surface of Spy. In these snapshots, the Im7 bound to Spy adopts conformations ranging from an unfolded conformation to a native-like structure (49).

CHAPERONES INVOLVED IN OUTER MEMBRANE PROTEINS BIOGENESIS

The outer membrane of *E. coli* contains mainly two types of proteins, lipoproteins and β-barrel membrane proteins. Since the biogenesis of lipoproteins and outer membrane proteins is the subject of recent reviews (52), we will restrict our discussion to the role that the periplasmic chaperones SurA, Skp, and DegP play in maintaining the solubility of outer membrane proteins as they transit the periplasmic space.

Skp

Skp is a <u>S</u>eventeen <u>K</u>ilodalton <u>P</u>eriplasmic protein (53). Initial evidence for Skp playing a role in the folding pathway of outer membrane proteins included its retention on an OmpF affinity column (54) and the observation that *skp* gene mutants can be isolated based on their increased sigma E activity (55). Skp is a nonessential periplasmic protein (54), and outer membrane protein levels are not significantly affected by deletion of *skp* (56). However, Skp-SurA double mutants are inviable, providing evidence that Skp and SurA function in parallel pathways (57). SurA depletion in a Skp null strain diminishes the levels of nearly all β-barrel proteins (56). Likewise, double mutants lacking both Skp and the periplasmic protease DegP are not viable at 37°C, possibly due to the accumulation of protein aggregates in the periplasm (58). SurA appears to participate in the primary pathway for outer membrane protein insertion, whereas Skp appears to provide a secondary pathway that is specifically necessary for some proteins such as LptD (59).

More than 30 proteins bind to Strep-tagged Skp, most of which are outer membrane proteins (56). Skp is involved in the handoff of substrates to the β-barrel assembly machine (BAM) complex, which helps to fold proteins and insert them into the outer membrane. Although Skp is primarily involved in the transport and folding of outer membrane proteins, it also interacts with some soluble periplasmic proteins including β-lactamase, OppA, and maltose binding protein (60), and its overexpression has been shown to be helpful for periplasmic recombinant expression of some proteins (61, 62).

Skp binds a variety of unfolded outer membrane proteins with binding affinities in the nM range, preventing their aggregation, and facilitates their insertion into negatively charged lipid membranes (63–65). Skp substrates include OmpA, LptD, and FhuA, and binding to these substrates occurs via aromatic residues and electrostatic interactions (66). Furthermore, biochemical experiments show that Skp selectively binds the newly synthesized outer membrane protein, PhoE immediately after its translocation across the inner membrane (67).

Evidence suggests that Skp functions in the periplasm as a molecular chaperone for several β-barrel proteins by maintaining soluble conformations before their insertion and folding into the outer membrane bilayer. Skp also sequesters partially folded intermediates of soluble recombinant proteins including single-chain antibodies expressed in the periplasm, preventing their aggregation (68).

The crystal structure of this highly basic protein reveals three long α-helical hairpins protruding from a central β-barrel structure that defines a trimerization domain (69, 70). This homotrimeric complex has the overall appearance of a jellyfish that resembles the cytosolic holdase chaperone prefoldin, with three flexible tentacles defining a large internal cavity that constitutes its substrate binding site (68, 69). It has been shown that unfolded outer membrane proteins bind inside this central, predominantly hydrophobic cavity by surface-surface interactions. However, electrostatic interactions between Skp and the outer membrane proteins, PagP, are important for PagP folding and outer membrane insertion (71). It has been postulated that successive formation of increasing electrostatic interactions allows substrates to climb into Skp's internal cavity (72).

The binding cavity of Skp has been estimated to accommodate substrate proteins up to ~25 kDa in size. However, Skp is able to interact with unfolded outer membrane proteins (uOMPs) that are much larger than this. NMR experiments on the ~35 kDa uOmpA bound to Skp showed that only the β-barrel domain of uOmpA is retained within the Skp binding cavity, providing an

explanation for how Skp interacts with larger uOMPs. Very recent kinetic and ion-mobility mass spectrometry data showed that the central cavity of Skp can actually expand to accommodate larger substrates, and that multiple Skp molecules can bind a single uOMP for larger uOMP substrates. Indeed, *in vitro* experiments revealed that 2:1 or higher Skp:uOMP stoichiometries are necessary for Skp to suppress the aggregation of larger outer membrane protein clients ([73](#)). Both the uOMP and the Skp arms are flexible, and small angle neutron scattering ([74](#)) and modeling studies indicate that the mobility of Skp's arms is important for allowing it to interact with a variety of conformations of the uOMP. After binding, however, Skp's arms become more rigid ([75](#)). NMR studies indicate that many weak interactions occur between the substrate and the chaperone; this, combined with the high mobility and flexibility of both binding partners, can help explain the ability of Skp to interact promiscuously with various substrates and different conformations of proteins. These interactions have the end effect of shielding the aggregation-prone uOMP from the aqueous environment ([52](#)). In addition to preventing aggregation, it has been proposed that Skp traps folding intermediates, stabilizes dynamic unfolded states of its substrates, and funnels them toward their native conformations ([76](#)).

SurA

SurA is a 45-kDa protein that was originally identified as being required for survival of *E. coli* in stationary phase, but not during log phase ([77](#)). The physiological defects of *surA* mutants are indicative of outer membrane perturbations, but are more severe than those of *skp* mutants, suggesting that SurA plays a more dominant role as an outer membrane protein chaperone ([55](#), [78](#), [79](#)). These defects include hypersensitivity to bile salts, detergents, and hydrophobic antibiotics, and reduced levels of outer membrane porins and reduced virulence ([80](#), [81](#)). In *surA* mutants, unfolded monomeric species accumulate in the folding pathway of the maltoporin LamB ([79](#)). Deletion of SurA also leads to major decreases in the abundance of outer membrane proteins in *Yersinia enterocolitica*, which in turn substantially affects the virulence of this pathogen ([82](#)). Differential proteomics showed that a number of outer membrane proteins are less abundant in a *surA* mutant strain, although most of them are less abundant due to activation of the sigmaE stress response, which lowers the expression of outer membrane proteins in the *surA* mutant ([83](#)). The decrease in abundance correlates with decreased mRNA levels for all the outer

membrane proteins except FhuA and LptD, suggesting that SurA is directly important for the folding of these two outer membrane proteins ([83](#)). The *lptD* gene actually shares an operon with *surA*, and overexpression of LptD in a strain lacking SurA fails to recover wild-type levels of LptD. Furthermore, these two outer membrane proteins show the most dramatic reduction in protein levels when SurA is depleted in a *skp* mutant strain. Although decreases at the RNA level appear sufficient to explain the decreases at the protein level for many outer membrane proteins, this does not necessarily eliminate the possibility that SurA plays an important role in the transport and folding of outer membrane proteins. Indeed, studies suggest that SurA does bind to the majority of outer membrane proteins during their transit through the periplasm, and SurA is essential for *in vitro* insertion of membrane proteins in a reconstituted system.

The *surA* gene is nonessential. However, like *skp* ([84](#)), a combination of *surA* and *degP* null mutations exhibits a synthetic lethal phenotype. This suggests that Skp and SurA may provide redundant chaperone functions in the periplasm ([84](#), [85](#)). This redundancy may explain why SurA mutants reveal relatively few obligate substrates. SurA cooperates with the disulfide isomerase DsbC in the folding of the outer membrane protein LptD ([11](#)). However, most models propose that SurA participates in the major pathway for outer membrane protein export and insertion, whereas Skp and DegP are involved in an auxiliary pathway ([86](#)). Further evidence for the existence of multiple parallel pathways for the folding and degradation of periplasmic proteins comes from an extensive genetic analysis conducted by the Ehrmann laboratory ([57](#)), where they individually, or in combination, mutated 15 different folding factors and proteases. All 7 different folding factors tested (SurA, DsbA, DsbC, FkpA, DegP, Skp, and PpiD) were synthetically lethal under at least one growth condition when combined either with each other or with a protease. A number of combinations also caused induction of the *spy* gene, which indicates activation of the periplasmic stress response, or leakage of β-galactosidase into the media, indicating a fragile cell envelope.

The primary structure of SurA is made up of four separate regions: an N-terminal region of ~150 residues, two peptidyl prolyl isomerase (PPIase) domains of ~100 residues each, and a short (~40 residues) C-terminal extension. Although SurA exhibits low PPIase activity *in vitro*, the PPIase domains are nonessential: a variant of SurA in which the N-terminal region is directly fused to

the C-terminal region without either of the two PPIase domains has *in vitro* chaperone activity and can almost entirely complement the *in vivo* phenotype of a *surA* deletion mutant, with the exception of novobiocin sensitivity ([87](#)). The two PPIase domains may, however, play a role in regulating the function of the chaperone domain ([88](#), [89](#)). The N-terminal structural domain has a deep elongated crevice that could accommodate unfolded polypeptides, suggesting a binding site for unfolded outer membrane proteins ([90](#), [91](#)). Consistent with this observation, SurA is known to bind several peptides with alternating aromatic amino acid motifs (Ar-X-Ar) frequently found in the sequences of outer membrane proteins ([91](#), [92](#)). Puzzlingly, crystallographic data showed that peptides containing these motifs actually bind to the first PPIase domain, not the chaperone domain ([93](#)); it is unclear if this binding to the first PPIase domain is relevant to SurA's function as an outer membrane protein chaperone. It is also still unclear how SurA binds to unfolded outer membrane proteins to prevent their aggregation, because it seems to lack the ability to encapsulate substrates in the way that Skp does. Making SurA more rigid by introducing disulfide bonds does not negatively affect its function, implying that flexibility is not as important for SurA as it is for Skp ([94](#)). However, both Skp and SurA share an ability to interact with substrates in a flexible dynamic fashion ([95](#)). Recent single-molecule force microscopy experiments suggest that SurA facilitates the insertion of β-barrel outer membrane proteins into the lipid membrane one β-hairpin at a time until the entire outer membrane protein has been inserted ([76](#)). There is currently no evidence that SurA is involved in the folding of soluble periplasmic proteins.

PROLINE ISOMERASES

Peptidyl prolyl isomerases (PPIases) catalyze *cis/trans* proline isomerization, an important step in protein folding. Most proline residues are in the *trans* configuration, but some proteins require prolines in the *cis* configuration in order for them to adopt their native structure. Proline isomerases interconvert proline *trans* and *cis* isomers on a biologically meaningful timescale. Four enzymes with PPIase activity exist in the periplasm: PpiA, SurA, FkpA, and PpiD. Since isomerization of proline residues between the *cis* and *trans* configuration is often a rate-limiting step in protein folding, it is logical that the proline isomerization activity of these proteins should be important for protein folding. Perplexingly, this has been difficult to directly establish *in vivo*. For example, a quadruple mutant that lacks all four PPIases in *E. coli* shows a defect in pilus formation but is still viable ([96](#)).

The first proline isomerase, PpiA, is a cyclophilin homologue. Like many folding factors, PpiA is induced by the Cpx regulatory system ([97](#)). However, null mutants in *ppiA* have normal levels of periplasmic and outer membrane proteins and no detectable phenotype ([98](#)), raising questions about PpiA's role in protein folding.

The second proline isomerase, SurA, clearly plays an important role in outer membrane protein folding, as discussed above. In addition to its chaperone function, SurA also contains two PPIase domains that show PPIase activity *in vitro* ([89](#)). However, the role of SurA's PPIase activity is unclear: mutants that disrupt SurA's PPIase catalytic activity have little impact on its *in vivo* activity in outer membrane protein insertion ([99](#)). Instead, it is the peptide binding activity of this PPIase domain that seems to be important for SurA's chaperone activity. Synthetic constructs made by inserting the chaperone domain of the unrelated chaperone, SlyD, into the second PPIase domain of SurA generates enzymes that are very effective in catalyzing proline isomerization, suggesting that the PPIase domains of SurA are not optimized for PPIase activity ([100](#)).

The third proline isomerase, FkpA, has a domain related to the FKBP family of prolyl isomerases as well as a chaperone domain ([101](#)). FkpA assists in the folding of recombinant proteins ([102](#)) and a MalF-LacZ fusion protein expressed in the periplasm ([103](#)). Furthermore, when overproduced, FkpA can rescue the lethal phenotype of a Δ*surA* Δ*skp* strain, suggesting that FkpA may be involved in outer membrane proteins biogenesis. It is not just the chaperone activity of FkpA that is important; the PPIase activity of FkpA has been shown to improve the proper periplasmic folding of colicin M ([104](#)). Although FkpA appears to only play a minor role in outer membrane protein biogenesis at 37°C, its importance increases with increasing growth temperature: FkpA function is as important as SurA for *E. coli* survival at 44°C ([105](#)). The binding rate and affinity of FkpA for outer membrane proteins increases under these conditions, suggesting that FkpA may be a temperature-responsive chaperone/PPIase for outer membrane protein biogenesis.

PpiD, like PpiA, is a periplasmic parvulin homologue. PpiD is anchored in the inner membrane by one trans-

membrane α-helix, and its parvulin-related PPIase domain extends into the periplasm. This PPIase domain, however, seems to be inactive (106–108). Overall, the PpiD protein does play a role in envelope biogenesis, because PpiD mutants are SDS/EDTA sensitive, indicating a cell wall defect. PpiD mutants, when combined with degP mutants, are, in addition, both temperature sensitive and salt sensitive (57). PpiD overproduction can rescue the lethality of a skp surA double mutant and the growth phenotype of a fkpA, ppiD, surA triple mutant in a way that is independent of its PPIase domain. Presumably, these effects are due to PpiD's reported chaperone activity (109).

In summary, although E. coli possesses multiple periplasmic proteins with prolyl isomerase domains, the importance of these proteins appears to be more dependent on their chaperone activity than their PPIase activity.

DegP

degP (also known as htrA for high-temperature requirement) was first identified as a gene essential for growth at elevated temperatures where protein unfolding/misfolding is more pronounced. DegP is upregulated by the sigmaE and Cpx envelope stress response systems. As mentioned previously, deletion of degP shows a synthetically lethal phenotype when combined with either a surA or skp mutant, suggesting that DegP plays a role in outer membrane protein biogenesis. However, DegP is well known for its role as a serine protease in the periplasm towards unfolded proteins; degP null mutants accumulate misfolded proteins. However, DegP has also been reported to have chaperone activity, which is evident at temperatures below 28°C, whereas its protease activity dominates at higher temperatures (110). Structurally, DegP has an N-terminal chymotrypsin-related protease domain that contains an active site His-Asp-Ser motif and two PDZ domains at the C terminus. PDZ domains are often involved in protein-protein interactions. DegP forms large ring-shaped 12-mers or 24-mers that can encapsulate bound substrates. This encapsulation is thought to be important for preventing its substrates from interacting with solvent (111), similar to how Skp prevents aggregation of its substrates by sequestration. However, formation of 12-mers/24-mers is not required for protease function because a variant that is trimeric still shows proteolytic activity (112). Outer membrane proteins bound to DegP show a remarkable resistance to proteolysis compared to misfolded model substrates. (DegP-bound outer membrane proteins remain stable longer than the 30 minutes used in the assay.) Crystallographic and electron microscopy data revealed that outer membrane proteins encapsulated by DegP adopt a structured conformation within the DegP ring, perhaps explaining why they are not degraded by DegP. Although cage formation is not essential for proteolytic activity (112), mutations that interfere with the formation of cage-like structures result in rogue proteases, suggesting that cage formation provides a layer of protection against indiscriminate proteolysis (113). DegP has also been reported to form bowl-like structures on lipid membranes (114).

As a chaperone, DegP enhances the in vitro refolding yield of the E. coli periplasmic protein MalS at 28°C; refolding yields are similarly enhanced for the nonnative substrates citrate synthase and β-galactosidase. DegP has also been shown to suppress the aggregation of the nonnative substrate lysozyme. In addition, the temperature sensitivity of DegP deletions can be rescued by the overproduction of a protease-deficient DegP variant (115, 116), consistent with it having chaperone activity. Moreover, a protease-deficient DegP variant can rescue the lethality caused by the expression of the autotransporter virulence protein pertactin (117). Strikingly, heterologous overproduction of the sHSP chaperone CeHSP17 from Caenorhabditis elegans is able to overcome the temperature sensitivity of a DegP deletion, allowing growth of wild-type E. coli to temperatures as high as 50°C, which is about 3.5°C above the normal maximal growth temperature of E. coli (118). It is not immediately obvious if this eukaryotic protein compensates for the chaperone or for the protease activity of DegP. However, since CeHSP17 is a chaperone with no known protease activity, it is more likely that it compensates for DegP's chaperone activity than its protease activity.

Results from the Fleming group suggest that DegP functions mainly as a protease during outer membrane protein biogenesis and that its chaperone activity does not significantly contribute to its role in OMP biogenesis (119). Although further work may eventually resolve the chaperone/protease DegP controversy, it is worth noting that the distinction between chaperones and proteases is ill defined and substrate dependent. Both chaperones and proteases need to interact transiently with substrate proteins to achieve their function. While cleavage of the substrate is a clear hallmark of proteases, if a protein lacks the cleavage recognition sequence for the protease, this resulting transient interaction may act to stabilize

the substrate or affect its folding. The AAA+ family of proteins, for instance, plays roles in both folding and degradation, which depend on the presence of additional proteins and the substrate used ([120](#)). The distinction between chaperone and protease activities for DegP could depend on how well DegP binds a particular substrate and how quickly (or slowly) it proteolyzes that substrate under various environmental conditions. Furthermore, the dramatic changes in oligomerization state of DegP that occur could be used to regulate whether DegP acts as a protease or a chaperone, depending on the needs of the cell.

DegQ

There are three related members of the HtrA protein family in *E. coli*: DegP, DegQ, and DegS. All three contain both chymotrypsin-like protease domains and at least one PDZ domain and all form oligomers ([121](#)). Like DegP, DegQ is proposed to have both protease and chaperone activity. Evidence suggests that both DegP and DegQ switch between a hexameric resting state and higher-order oligomers in the presence of substrate ([114](#), [122](#)–[124](#)). A crystal structure of a DegQ-lysozyme complex has been obtained that consists of 12 DegQ molecules arranged in a cage surrounding 5 to 6 lysozyme molecules ([125](#)). Protease-deficient mutants of DegQ exhibit weak chaperone activity in refolding MalS ([125](#)). The structural similarities and differences between the DegQ structure and the GroEL/S double-donut structure have been described ([122](#)).

COMPARISON BETWEEN THE MECHANISMS OF PERIPLASMIC AND CYTOSOLIC CHAPERONES

It is interesting to more generally consider the similarities and differences between ATP-dependent cytosolic chaperones and ATP-independent periplasmic chaperones. Chaperones play important roles in the folding of proteins under both normal and stress conditions in both the periplasmic and cytosolic compartments. Chaperones in both compartments need to deal with similar issues, like the tendency of exposed hydrophobic residues to aggregate and the need to keep proteins in an at least partly unfolded secretion competent form. It is thus not surprising that some of the overall principles of chaperone action are shared between cytosolic and periplasmic chaperones. In the cytosol, hydrophobic residues generally end up buried within the hydrophobic core of the protein itself. In the periplasm, hydrophobic surfaces necessary for outer membrane proteins to be integrated into the outer membrane make them particularly aggregation prone while in an aqueous environment like the periplasmic space. To sequester hydrophobic residues, both cytosolic and periplasmic chaperones engage in encapsulation strategies, for example, within a closed chamber for the cytosolic chaperone GroEL or buried within the tentacles of the jellyfish-like periplasmic chaperone Skp.

One big difference is the lack of ATP in the periplasm. Many, but not all, cytosolic chaperones utilize ATP binding or hydrolysis to drive substrate binding and release. Those cytosolic "holdase" chaperones that do not use ATP generally work in concert with ATP-dependent chaperones. Without the driving force of ATP, periplasmic chaperones need to have much more finely balanced affinity constants for binding and release than cytosolic chaperones do. Otherwise, the chaperone either runs the risk of binding too tightly to its substrate, and never releasing it, or if it has a substantially higher affinity for the unfolded than the folded state, it will unfold the protein substrate by dragging the equilibrium in this direction. It has been hypothesized that the affinity of outer membrane proteins for the lipid environment provides the thermodynamic gradient that drives the transport of outer membrane proteins and substitutes for the need for ATP ([12](#), [126](#)).

It is not absolutely necessary for each step in a chaperone cascade to be down a thermodynamic gradient, as long as the overall gradient is downhill. The use of ATP binding and hydrolysis in cytosolic chaperones allows for major shifts in binding affinity, allowing binding and release of a wide variety of substrate proteins. Periplasmic chaperones lack this resource and thus, in general, would need to more delicately balance substrate binding and release. This would appear to pose a problem for periplasmic chaperones that have broad substrate specificity, because one would expect that different substrates would bind with different affinities. This consideration may contribute to the higher prevalence of substrate-specific chaperones in the periplasm than in the cytosol. As previously mentioned, periplasmic chaperones can be present in astonishingly high concentrations. For example, Spy's concentration following tannin or butanol stress approaches or exceeds 2 mM, and constitutes approximately 40 to 50% of the total protein content of the periplasm. This may be a reflection of simple mass action considerations. Spy's mode of action involves very rap-

idly binding folding intermediates and allowing them to fold while bound to the chaperone. This rapid binding is dependent on the K_d of the interactions and the concentrations of the interacting partners. Since there is no possibility of ATP driving release, the affinity of the interaction cannot be too high, because this would prevent release of the substrate. Given this limitation, evolution has apparently chosen to ramp up the concentration of Spy after stress to very high levels likely to ensure rapid binding of folding intermediates so that Spy can effectively compete against aggregation. It may also reflect a high demand for chaperone activity following butanol or tannin stress that may induce widespread protein unfolding.

The stresses leading to induction of both periplasmic and cytosolic stress-induced chaperones overlap to some extent. Heat shock is the classical inducer of cytosolic chaperones, and extreme heat shock is an inducer of the sigmaE response, which in turn controls the chaperones, proteases, and outer membrane assembly factors BamA, BamC, BamD DegP, DegQ, DegS, DsbC, FkpA, GroL, and SurA (14). Ethanol and overproduction of unfolded proteins induce stress responses in both compartments (24, 127). Certain stresses, such as exposure to tannins or low pH, appear to specifically induce periplasmic stress responses (16, 31).

OTHER CHAPERONES

A number of other periplasmic proteins have been identified as having chaperone activity *in vivo* through a fold-or-die selection: OsmY, OppA, DppA, and Ivy (128). All four of these proteins can stabilize an unstable version of maltose binding protein in a fusion biosensor similar to the one that was used to discover Spy. These proteins were also shown to have *in vitro* chaperone activity in inhibiting the aggregation of a number of unrelated client proteins. OsmY is a highly soluble protein of unknown function that is upregulated by several different protein folding stress conditions, including osmotic stress. It is also very effective as a solubility-enhancing fusion tag, consistent with it having chaperone function. OppA and DppA are oligopeptide-binding proteins that facilitate the import of peptides into the cytosol. OppA has a preference for binding positively charged peptides (129), and DppA was reported to have chaperone activity *in vitro* (130). Their ability to bind peptides may allow them to bind unfolded proteins and therefore operate as chaperones. Ivy is a potent inhibitor of vertebrate lyso-

zyme. However, deletion of *ivy* does not make *E. coli* more susceptible to hen egg white lysozyme, suggesting that Ivy may have other functions, consistent with its reported chaperone activity. Ivy, the sHSP chaperone IbpA, and the cytosolic chaperone GroEL are all induced in cultures that produce recombinant human growth hormone (131). It remains to be determined how the reported chaperone activity of OsmY, OppA, DppA, and Ivy (128) are related to their as yet poorly defined biological functions.

CHAPERONES INVOLVED IN THE FOLDING OF EXTRACELLULAR PROTEINS

A number of large filamentous structures exist on the surface of *E. coli*. These include pili, fimbriae, and curli. The polymerization of the major subunits in these structures is a facilitated process that, for pili and fimbriae, involves β-strand transfer. This is a structurally well-defined and elegant mechanism that neatly allows one protein to facilitate the folding of another without becoming part of its final structure. For type I fimbriae, the structural subunits of the fimbriae are called FimH, G, F, and A. All subunits contain an incomplete immunoglobulin type fold that are missing a single β-strand and thus expose a hydrophobic groove. Instead of promoting aggregation, this groove is filled by a β-strand donated by the dedicated chaperone/holdase FimC during transport through the periplasm. This process is called donor strand complementation. After transport through an outer membrane pore formed by the FimD usher, the β-strand of FimC is replaced by the β-strand of a previously transported structural subunit. This allows the structural subunits to polymerize in a linear series. Binding of the structural subunit-FimC heterodimer to the FimD usher opens the hydrophobic groove of the structural subunit and brings it into close proximity to another structural subunit, enabling polymerization through β-strand exchange between the two structural subunits. Other fimbriae and pili assemble by very similar mechanisms.

Curli are long extracellular polymers that are involved in biofilm formation, adhesion, and host invasion (132). They are composed of CsgA, the major structural subunit, and CsgB, a minor structural subunit, that together in a ∼20:1 ratio (133) form amyloid polymers that are functional (in contrast to most other amyloids, which are pathological). The curli fibrils form a tangled mess of thin threads that are the major constituents of the matrix

in curli-associated biofilms (134). The chaperones involved in curli formation are CsgC, CsgE, and CsgF. CsgC performs a role somewhat analogous to that of the SecB cytosolic chaperone: namely, to keep the subunits in a secretion competent, partially unfolded configuration (132, 135, 136). *In vitro*, CsgC is extremely efficient at preventing CsgA polymerization, working in ratios as low as 1:500 (136). The chaperone Spy can also inhibit CsgA polymerization, but Spy is much less effective than CsgC (137). CsgE can also inhibit CsgA polymerization. It acts as a secretion adaptor to target CsgA to the outer membrane pore, which is composed of CsgG subunits. A nonamer of CsgE serves as a capping adaptor that transiently blocks the CsgG secretion channel (138). CsgF also forms a complex with the CsgG secretion channel. In contrast to the transient association of CsgE with the CsgG channel, CsgF is a constitutive component of the channel. In the absence of CsgF, CsgB can no longer function as a curli initiator and CsgA fails to polymerize.

Thus, CsgF probably acts as a chaperone that couples CsgA secretion to polymerization (139). Following transit through this pore, CsgA and CsgB polymerize into cross-β-strand amyloid structures (see Fig. 1). Like fimbriae, these curli fibers are β-sheet-rich cross-β-strands. However, unlike fimbriae, polymerization of curli fibrils is not known to involve strand exchange between subunits. Fimbriae and curli also differ in the cellular location where they polymerize: fimbriae polymerize within the periplasm, whereas curli polymerize in the extracellular space (140). This is possibly to avoid the intracellular toxicity of amyloid assembly intermediates that are generated in curli formation (132).

ROLE OF PERIPLASMIC CHAPERONES ON THE EXPRESSION OF RECOMBINANT PROTEINS

There have been a number of attempts at enhancing the expression of recombinant proteins in the periplasm by
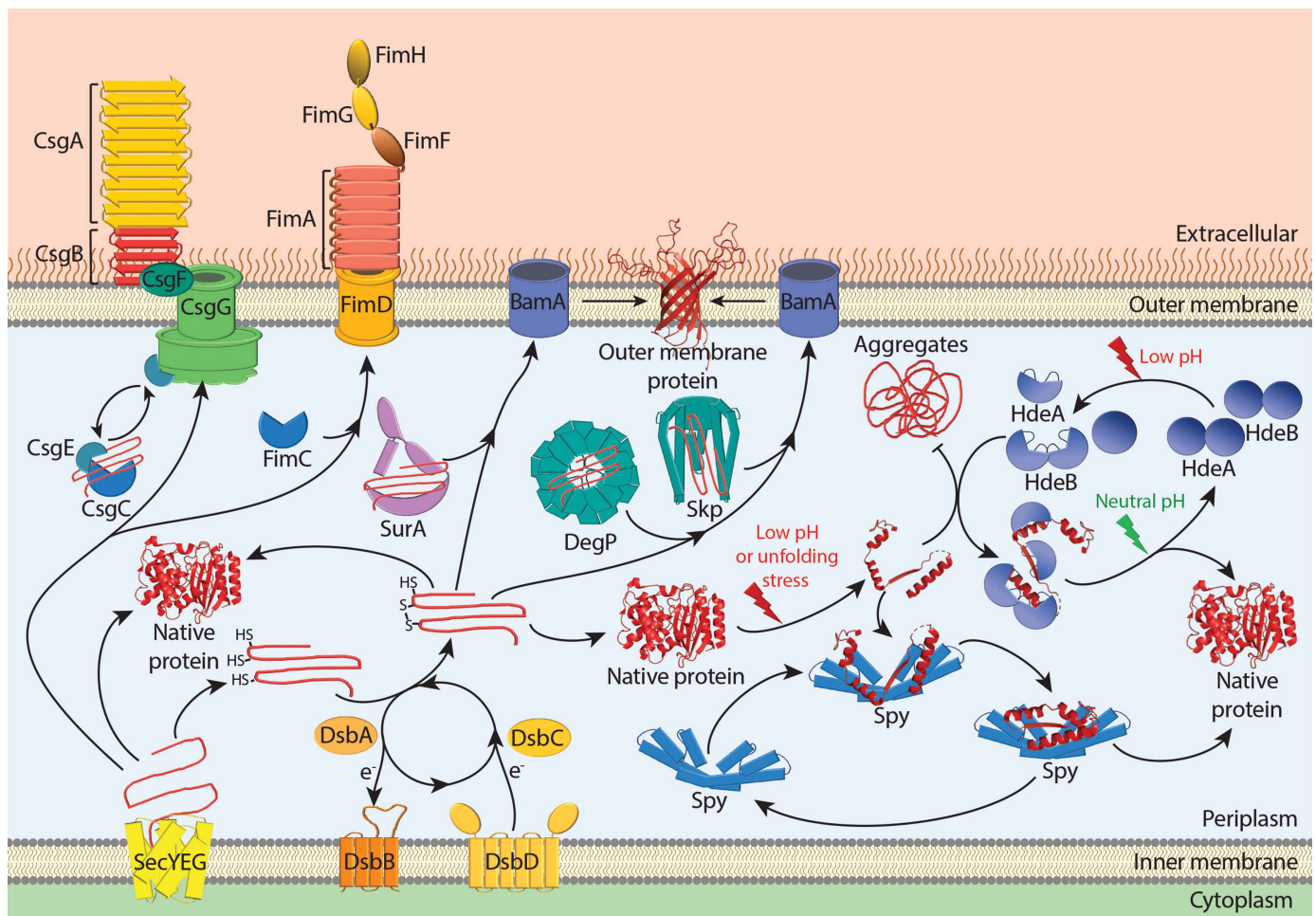

Figure 1 An overview of the chaperones and folding factors affecting protein folding in the periplasm of *E. coli*.

overexpression of periplasmic chaperones and folding catalysts. For example, a plasmid that simultaneously overexpresses DsbA, DsbC, FkpA, and SurA has been constructed and successfully used for this purpose ([141], [142]). However, this approach may not be a panacea for all expression issues. As discussed above, *E. coli* naturally responds to the presence of misfolded proteins in the cellular envelope by overexpressing periplasmic chaperones using the SigmaE, Cpx, and Bae regulatory systems. Given the likelihood that chaperones are naturally induced in response to recombinant protein overexpression and that our current grasp of chaperone-substrate specificity is poor, chaperone coinduction may not universally improve the folding of all overexpressed recombinant proteins. However, the folding environment of the periplasm can be optimized to improve the folding of specific proteins using folding biosensors. These biosensors link the *in vivo* stability of specific proteins to a genetically selectable marker, such as antibiotic resistance. Coupling chromosome mutations with a demand for high antibiotic resistance in strains containing these folding biosensors results in superfolder bacteria strains that are customized for promoting the expression and folding of specific unstable proteins. Applications of these folding biosensors have uncovered previously unrecognized periplasmic chaperones: Spy, OsmY, Ivy, DppA, and OppA. Given the wide range of potential protein folding problems faced by various proteins, it is not surprising that *E. coli* selects unique chaperone overproduction solutions for individual protein folding problems.

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